INHIBITION OF ADENOVIRUS IN VITRO DNA REPLICATION BY VESICULAR STOMATITIS VIRUS LEADER RNA

1986

REMENICK

1. REPORT DATE **AUG 1986**	2. REPORT TYPE **N/A**	3. DATES COVERED **-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Inhibition of Adenovirus in vitro DNA Replication by Vesicular Stomatitis Virus Leader RNA	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Uniformed Services University Of The Health Sciences Bethesda, MD 20814**	8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release, distribution unlimited**

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT **SAR**	18. NUMBER OF PAGES **155**	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**			

APPROVAL SHEET

Title of Thesis: Inhibition of Adenovirus in vitro DNA Replication by Vesicular Stomatitis Virus Leader RNA
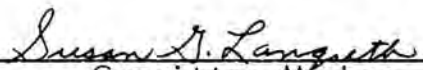
Name of Candidate: James Remenick
Doctor of Philosophy Degree
August 18, 1986

Thesis and Abstract Approved:
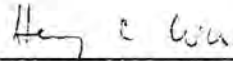
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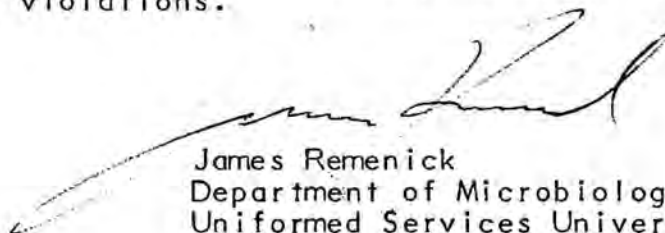
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ii

To Mom and Dad with Love

ACKNOWLEDGEMENT

Table of Contents

ix

List of Tables

List of Figures

Introduction

History

The members of the virus family, Rhabdoviridae (from Greek rhabdo = rod), infect large numbers of vertebrates, invertebrates and plants (Wagner et al., 1984). Many species have been isolated from humans, domestic animals, wildlife, fish, crabs, reptiles and insects. Most members are quite prolific and capable of crossing phylogenetic barriers infecting both plants and insects or insects and animals. This ability led Johnson et al. (1969) to speculate that rhabdoviruses were originally a plant pathogen able to replicate in insects that eventually infected animals.

Some well studied members include sigma virus of the fruit fly, spring viremia virus of carp, bovine ephemeral fever virus, vesicular stomatitis virus of cattle, swine and horses, Lagos bat virus and rabies virus which infects all warm blooded animals (Brown and Crick, 1979). These viruses are members of the same family based on the following characteristics. They contain a nonsegmented, anti-message sense, single-stranded RNA genome, which encodes 4-5 genes, possess a lipid envelope and have a bullet or conical shape as determined by electron microscopy. The two major genera that infect warm blooded animals based on serological interrelatedness are the rabies virus group or lyssaviruses (from Latin lyssa = madness) and the vesicular stomatitis virus group or vesiculoviruses (from Greek vesica = bladder, blister) (Table I).

Within lyssaviruses is the virus which produces the dreaded disease, rabies (from old Sanskrit rabhas = to do violence). Rabies is one of the oldest recorded afflictions of humans and animals with

Table I. Rhabdoviruses of Warm Blooded Animals.

A. Lyssavirus genus

    1. Rabies, Mokola, Duvenhage, Lagos bat virus

    2. Obodhiang

    3. Katonkan

B. Vesiculovirus genus

    1. Indiana, Cocal, Alagoas

    2. New Jersey

    3. Piry, Isfahan, Chandipura

C. Minor serogroup

D. Ungrouped serotypes

Shown are an assortment and categorization of some of the members of the rhabdoviruses that infect warm-blooded animals. Members of the lyssavirus and vesiculovirus genera were named for the locations at which they were originally isolated.

reference dating back to 2000 B.C (Miller and Nathanson, 1977; Tierkel, 1971). It is also one of the earliest recognized communicable diseases. The Greeks and Romans were the first to affiliate rabies of animals to the disease in humans, and the Romans named the infectious material, virus (Latin for poison) (Steele, 1975). Celcus, a Roman doctor recommended in 100 A.D. that wounds inflicted by a rabid animal be cauterized with a hot iron to kill the "virus". This remained the treatment of choice until 1885. In that year, Pasteur (1885) reported the development and successful testing of the first rabies vaccine. A nine-year-old boy who had been bitten 14 times by a rabid dog was given 13 inoculations over a ten-day period of dried spinal cord preparation which had been removed from rabbits that died after injections of rabies virus. The boy survived the dog bites and also the inoculations which were known to contain large numbers of virulent virus. By 1886, 2490 persons had been inoculated after exposure with a 90% success rate (Pasteur, 1886). Since then, many variations of the vaccine have been developed which are much more protective and much less harmful (Ginsberg, 1980). Despite the availability of the vaccine, rabies is still prevalent today and infections are almost always fatal if treatment is not administered immediately after contact. Other members of the lyssavirus group, Mokola, Duvenhage and Katonkan were more recently isolated in Africa (Brown and Crick, 1979). They are serologically related to rabies virus, but only Mokola and Duvenhage have been associated with clinical diseases in man, each causing a fatal brain infection.

The vesiculovirus genus has three serogroups, each named for the location at which they were isolated (Brown and Crick, 1979). The

Piry and Chandipura viruses were isolated from South America and India, respectively. Each can be found in both man and domestic animals obliged to the region. These viruses are not fatal, producing only fever with chills and headaches in the infected individuals which subsides in 1-5 days. Vesicular stomatitis virus (VSV) serotype Indiana has been extensively studied and is called the prototypical rhabdovirus. Pigs, cattle and horses are the most common hosts of VSV which is a disease basically restricted to the Western hemisphere.

Pathology of VSV

The clinical symptoms of VSV infection in the typical ungulate are vesicle formation of the skin in restricted areas near the coronary band of the feet and in the mouth with a fever that quickly subsides after rupture of the vesicles (Blood and Henderson, 1974). These symptoms are often misdiagnosed as hoof and mouth disease, a more severe picornavirus infection which is solely differentiated from VSV by a more generalized vesicle formation of the animal's body (Cottral et al., 1970). VSV infection is not fatal but can cause serious problems as it did in 1862 during the Civil War when an epidemic broke out among the cavalry of the Union Army a few days before battle (McClellan, 1862).

Today, VSV infections are more important from an economic point of view in the care and raising of domestic animals. VSV spreads quickly by direct contact, contaminated water and possibly insect vectors (Brown and Crick, 1979). An entire herd could be infected in a very short time which leads to extra feed costs, lost earnings, veterinary bills and the loss of animals. An outbreak of VSV in the San Joaquin Valley of California resulted in a loss of $225,000 to two dairies over

a two-month period (Goodger et al., 1985). While VSV itself is not fatal, blisters on the legs are prone to secondary bacterial infections which often lame an animal permanently (Blood and Henderson, 1974). In spite of these complications, subclinical infections are common. Neutralizing antibodies to VSV have been found in many animals in the absence of any signs of previous infection. VSV is fatal to one species. Only inbred, neonatal hamsters are sensitive to VSV, and as little as 10^5 particles can kill (Fultz et al., 1981). Consequently, these animals have been used as in vivo models of VSV infection.

Humans appear to be incidental if not dead end hosts of VSV infection. The symptoms of a typical VSV induced human illness will range from inapparent to flu-like which pass quickly. At its worst, VSV may infect the head area beginning as keratoconjunctivitis and lead to encephalitis. The affected individual develops a severe headache which passes in a few days. Two laboratory researchers, Dr. Craig R. Pringle and Dr. Robin Robinson infected themselves with VSV in two separate incidents. Dr. Pringle accidentally cut himself with glass fragments from a broken bottle of VSV infected cells (personal communication). He developed a very high antibody titer to VSV with fever and chills but recovered in two weeks. Dr. Robinson inhaled large amounts of VSV while working at a sterile exhaust hood (personal communication). Within 24 hours, he experienced severe back pain, headache with fever and swelling around the eyes. He recovered fully in 10 days.

Structure and Life Cycle

The virus particle is composed of 65-75% by weight of virus derived protein, 15-20% by weight of host-cell derived lipid, 3% by weight carbohydrate and 1-2% by weight of noninfectious, nonsegmented,

Figure 1. <u>Structure and Morphology of VSV</u>. Depicted are an electron micrograph of negatively stained VSV particles and a characterization of the cross section of a typical VS virion with the location of the structural components indicated. Visible in the electron micrograph are classical bullet shaped virions and a number of amorphous particles which are most likely virions that were damaged in preparation.

VESICULAR STOMATITIS VIRUS MORPHOLOGY

200,000 ×

NS PROTEIN

GENOME RNA
+
N PROTEIN

G PROTEIN

M PROTEIN

LIPID ENVELOPE
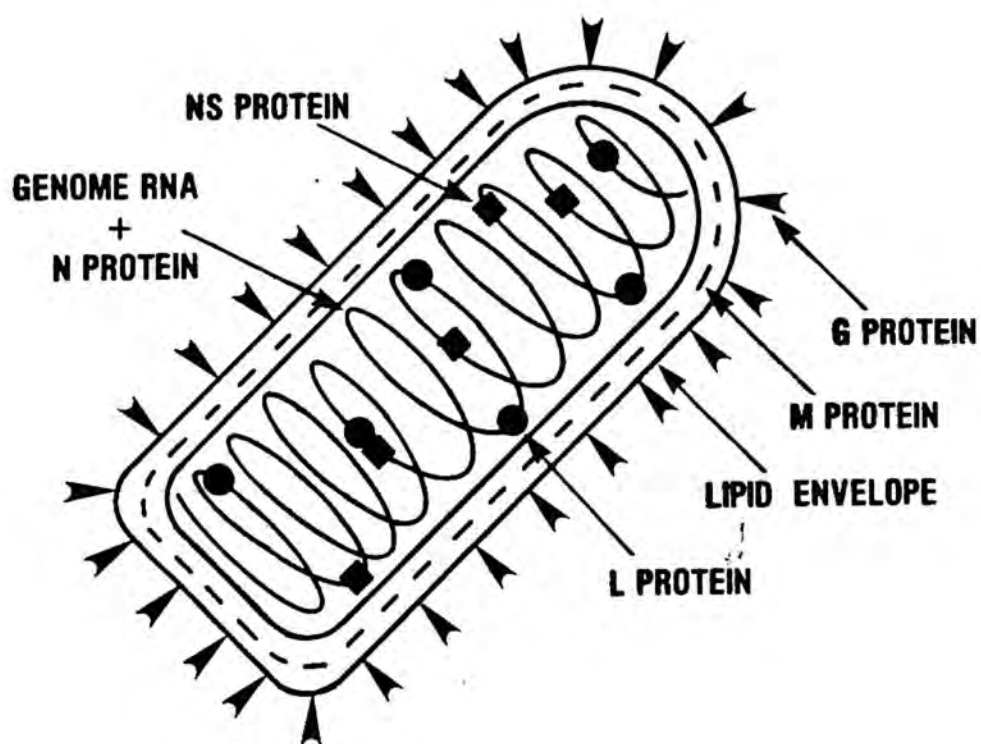
L PROTEIN

single-stranded RNA (Figure 1). VSV infections initiate by attachment of the viral glycoprotein, G, to the outer surface of the host cell as evinced by the ability of antisera generated against G protein to neutralize virus infectivity (Kelly et al., 1972; Wiktor et al., 1973). The virus particle enters the cell by receptor mediated endocytosis (Simpson et al., 1969). It has been proposed that VSV was able to gain access to L-cells by cell to virus envelope fusion (Heine and Schnaitman, 1971), but in retrospect, that observation was caused by centrifugation of virus-cell complexes on initial exposure and was not a natural or typical process of VSV infection.

In support of the envelope fusion model, however, McCoombs et al. (1981) compared the infectivity of VSV to cells treated with cytochalasin B, an inhibitor of endocytosis, with untreated cells. Infectivity was found to be equal suggesting that envelope fusion had occurred. Similar experiments using chloroquine, an inhibitor of lysosome function, also did not reduce VSV infectivity. Although viropexis is the generally accepted manner in which VSV enters the cell, alternative methods such as envelope fusion, or an as yet undiscovered process, cannot be ruled out.

The process by which the virion enters the cytoplasm is not clear. It is generally thought that lysozomes fuse with the VSV-containing vesicle releasing catabolic enzymes, lowering the pH of the vesicle which breaks down the virion and allows the nucleocapsid to pass through the vesicle envelope (Marsh et al., 1983). Simpson et al. (1969) found that at the site of viral attachment, the cell's plasma membrane thickens, presumably due to accumulation of protein. Within 5 minutes after absorption, 60% of the viral particles were found in

vacuoles. By 1 hour, nucleocapsids were no longer visible, and infection was underway.

Primary transcription begins immediately upon entering the cytoplasm (Figure 2) (Banerjee et al., 1977). The virion associated RNA dependent RNA polymerase transcribes a small 47 nucleotide (n.t.) RNA called leader RNA and five monocistronic mRNAs which unlike leader RNA are each capped and polyadenylated. The messages are selectively translated by the host cell machinery to the exclusion of host derived mRNA. Unlike the paramyxoviruses, VSV does not degrade host mRNA. Instead, VSV is believed to affect ternary complex formation of protein synthesis by limiting the availability of eukaryotic initiation factors 3 (eIF-3) and eIF-4B (Thomas and Wagner, 1983). Under these conditions, VSV messages may be better able than the host's to compete for ribosomal binding sites. Leader RNA does not code for any known protein and is not translated. Some molecules of leader are found in the nucleus of the infected cell where it has been suggested that they interfere with the processes of host cellular DNA and RNA synthesis (Kurilla et al., 1984).

Ultraviolet (U.V.) inactivation studies by Ball and White (1976) demonstrated that the gene order of VSV is leader, N, NS, M, G and L (Figure 3). In these studies, Ball and White in vitro transcribed the 42S VSV genome after irradiation with U.V. light. U.V. light will produce uridine dimers in the genome preventing RNA polymerase from transcribing past the point of dimerization. If the genes were transcribed independently by an RNA polymerase which initiated at several sites within the genome, inhibition of synthesis of each message would be directly related to the size of its respective gene.

Figure 2. <u>The Replication of VSV Within the Host Cell</u>. The negative stranded VS viral genome transcribes leader RNA and five capped and polyadenylated mRNA. After translation of the viral messages, the template is replicated, forming genome length positive stranded RNA. These positive strands are replicated into negative stranded genome by the viral polymerase, which are packaged with viral protein into infectious particles. DI particles are generated at this point when the polymerase for unknown reasons, falls off the positive stranded template, reattaches to the newly synthesized strand and continues synthesis of complementary RNA. The resulting RNA molecule contains only sequence information derived from the 5' end of the VSV genome, and its only transcription product is DI leader RNA which is derived from the 5' end 46 n.t. of the wild-type (wt) VSV genome. DI genomes are packaged with viral protein but are smaller in size than wt VSV and easily isolated.

VSV

(−) 3′ ⸻ I N NS M G L ⸻ 5′

PRIMARY TRANSCRIPTION

(−)
(+)

I 5 mRNA

PRIMARY REPLICATION

(−)
(+)

SECONDARY REPLICATION

(+)
(−)

(+)
(−)

INFECTIOUS
VIRUS

DI PARTICLES

12

Figure 3. <u>The VSV RNA Genome and Its Transcription Products</u>. The VSV
genome RNA is depicted in the 3' to 5' orientation with the number
and position of untranscribed nucleotides indicated in parenthesis.
The order, relative size (except leader RNA) and quantity (in percent)
of each transcription product (l = leader RNA; N, NS, M, G and L mRNA)
are shown with the RNA represented in a 5' to 3' orientation. Symbols:
(o) = the 5' cap of VSV mRNA molecules; (}) = the poly(A) tail of VSV
mRNA molecules.

VSV RNA Genome (11,162 nucleotides)

[3]   [2]   [2]   [2]   [2]   [59]

3'   5'

L
6%

G
8%

M
11%

NS
17%

N
24%

l
33%
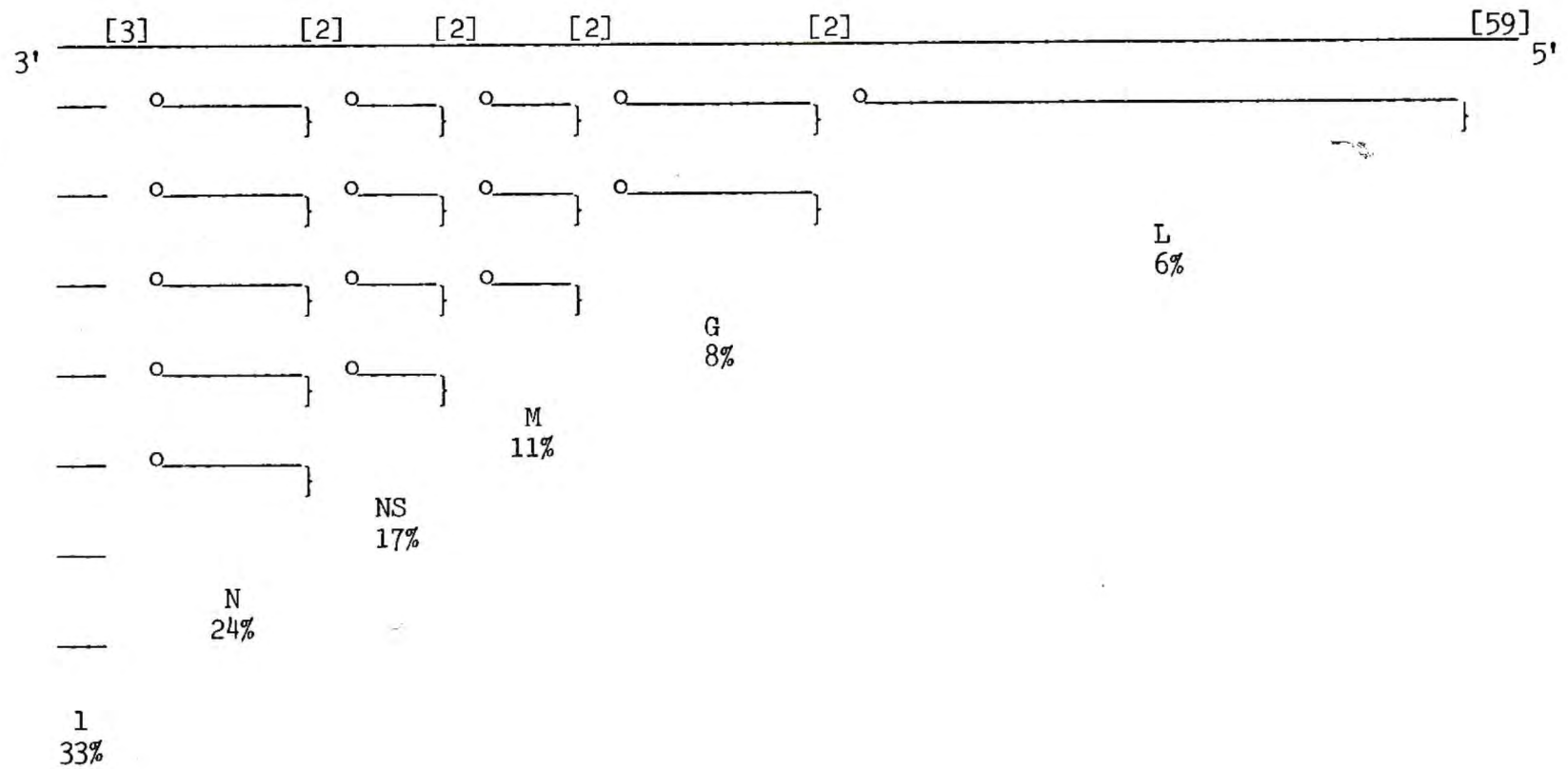
What they found, however, was that inhibition of transcription of each message was directly related to its distance from the 3' terminus of the genome and independent of the size of the gene. Emerson (1982) in vitro transcribed VSV nucleocapsids in the presence of the ribonucleotides ATP and CTP. She observed that only RNA products derived from the 3' terminus of the genome were produced and concluded that the RNA dependent RNA polymerase enters the RNA genome at the 3' end, and downstream sequences are only transcribed after the preceding sequences.

Based on these results, Iverson and Rose (1981; 1982) determined by hybridization of small DNA fragments to RNA of VSV infected cells that transcription along the VSV genome proceeds in an attenuated fashion. They observed a 30% decrease of transcription across each gene junction and concluded that the transcriptase enzyme pauses from 3 to 5 minutes at each of these junctions. They proposed that an attenuator may be responsible for pausing of the transcriptase, and pausing may increase the likelihood of the enzyme detaching from the template resulting in attenuated transcription.

Replication of the (-) strand into a full-length (+) strand begins at some time after primary transcription and is accomplished by the same viral RNA dependent RNA polymerase used during transcription (Figure 2). The switch from primary transcription to primary replication may be regulated by the availability of N protein in the cytoplasm. Blumberg et al. (1981) proposed that a viral attenuator exists within the VSV genome which prevents synthesis of full-length genomic sized RNA molecules from being made early. Accumulation of N protein later in infection binds to the genome at the attenuation site inactivating it, thus, allowing the viral polymerase to synthesize (+)

stranded genomic RNA molecules. Leader RNA may play a part in the mechanism of switch from transcription to replication by binding excess N protein made early during infection, thereby, delaying the suppression of the genomic attenuator until large quantities of N protein are built up within the cell. N protein-leader RNA complexes have been found within infected cells whereas viral mRNAs are typically unbound. Alternatively, but not to the exclusion of this hypothesis, leader RNA may simply contain the sequence required for N protein to attach and coat the genome (Keene et al., 1978).

From the (+) stranded molecules, cRNA is replicated which is packaged with the viral proteins, N, NS and L. G protein has accumulated at discrete regions of the cellular membrane, and M protein is somehow involved with getting the newly formed viral nucleocapsids to those sites. Once M and G proteins have associated with the nucleocapsid at the membrane, infectious virions bud out into the extracellular environment.

The exact functions of the viral proteins are not clear (Table II). All five may be involved in the regulation of transcription and replication of the genome. G protein, as stated previously, is the viral attachment molecule, and may function in assembly of virus particles in the infected cell by aggregating in specific areas of the cell membrane. M protein is the glue, so to speak, that holds the helical genome to the lipid portion of the particle. It may function during assembly to direct nucleocapsids to specific areas of the membrane. L protein is the viral RNA dependent RNA polymerase (also called transcriptase or replicase) because of its large size and similarity to the E. coli RNA polymerase (Emerson, 1976). From the in

Table II. VSV Proteins.

Viral Protein	Number of Molecules Per Particle	Size (Kd)	Function
N (nucleocapsid)	1000 - 2000	50	shielding/regulating
NS (phosphoprotein)	60 - 70	40	transcription/replication
M (matrix)	1600 - 4000	29	encapsidation/assembly
G (glycoprotein)	500 - 1500	70	absorption/penetration
L (polymerase)	30 - 35	190	transcriptase/replicase

Shown is a comparison of the distribution, sizes and functions of the five proteins of VSV. Although the functions listed have clearly been demonstrated, they are by no means the only operations performed by these viral proteins. All five are structurally required for assembly of the virion.

vitro studies of Wertz (1983), it is clear that L protein requires NS
and N protein for transcription and replication of the genome RNA. The
exact function of the NS protein is not known. It exists in at least
two forms, NS_1 and NS_2, which differ in their degree of phosphorylation.
It may be that NS holds the template in a position that allows L pro-
tein to begin transcription. N protein functions as a dimer and a
tetramer to completely coat the genome RNA. This may protect the
template from RNase attack within the cell and plays a role in the
regulation of viral transcription and replication. Despite the tight
binding of N protein, the RNA template is still transcribed by the
viral polymerase.

Host cell proteins may be involved with VSV replication (Wagner
et al., 1984). Kang et al. (1981) described the phenomenon of suppres-
sion of VSV defective interfering (DI) particle generation as a func-
tion of human chromosome 16. Host proteins have been isolated from
purified virions, and host range mutants have been described (Obijeski
and Simpson, 1974; Pringle, 1978). Although the function and identi-
fication of host cell proteins during the life cycle of VSV have re-
mained a mystery, it seems clear that they do play a part.

VSV generates DI particles during productive infection. These
particles contain only a portion of the viral genome, but all of the
structural proteins of the standard virion. DI particles are defective
in that they do not contain the genetic information to code for all five
structural proteins of VSV. They are consequently not able to self-
replicate, but when coinfected with wild-type (wt) VSV, are replicated
by the wt polymerase and greatly suppress replication of the helper
virus. The mechanism of this phenomenon, termed autointerference, is

not understood. It has been suggested that DI particle replication competes with the standard virus for the limited amount of viral polymerase and N protein available in the infected cell.

DI particles are generated by a mistake which occurs during viral replication (Figure 2). After transcription of the genome length (+) strand, the replicase then synthesizes full-length (-) strands. For unknown reasons, the replicase sometimes falls off the (+) stranded template after only synthesizing a portion of the genome. With the newly synthesized strand still attached, it then reattaches to the genome further downstream from its falling off point and continues replication. Alternatively, after the replicase falls off the genome, it reattaches to the newly synthesized strand and transcribes its complementary sequence creating a stem-loop structure. Despite the lack of an intact genome, DI particles are replicated efficiently and packaged into infectious particles which indicates that DI genomes contain all the information necessary for the initiation of replication at their 3' and 5' termini and for encapsidation into nucleocapsids. As stated above, the DI genome encodes the genetic information from the immediate 5' end of the standard genome. These sequences are identical to those found at the 3' termini of the standard virus for 18 n.t. and highly homologous for the next 40. Although the exact requirements for initiating RNA synthesis and encapsidation of the virus are not known, these highly conserved sequences are clearly essential.

DI particles are transcriptionally active during infection. Their sole transcription product is a 46 n.t. RNA coded from the exact 3' terminus of the DI genome which corresponds to the 5' terminus of the wt genome. This small RNA is not capped or polyadenylated like its

cousin wt leader RNA, has been referred to as the DI product, DI leader RNA, or the DI small RNA and is 60% homologous in sequence with wt leader RNA (Rao and Huang, 1979).

Cell Killing

VSV is the most scrutinized member of the rhabdovirus family. Much attention has been directed toward examining its ability to in-hibit macromolecular synthesis of the host cell and cause cell death. Early experiments, however, were not designed to distinguish cell death from the inhibition of cellular macromolecular synthesis, a separate, but related phenomenon. This introductory review will therefore dis-cuss the process of cell killing (CK) and macromolecular synthesis inhibition separately.

VSV is an excellent model system to study this phenomenon of CK because of its inherent simplicity. VSV is an uncomplicated virus as compared to other lethal viruses such as herpes simplex virus type 1 or adenovirus which both code for a large number of protein and RNA molecules. Many of the functions of the VS viral proteins have been determined, and a large number of mutations have been isolated. These mutants are grouped into five classes which are phenotypically iden-tified with specific VSV protein products; class I with L protein, class II with NS protein, class III with M protein, class IV with N protein and class V with G protein.

Marcus and Sekellick in 1974, when little of the molecular properties of VSV were known, quantitated VSV's CK ability. They developed the single cell survival assay to determine the number of infectious particles required to kill the host cell. Monolayers of vero cells were infected with VSV at various dilutions. After 60

minutes, presumably sufficient time for nucleocapsid entry, cells were trypsinized and dispersed into fresh media containing VSV antisera to prevent cross infectivity. The cells and cell-viral complexes were incubated and colony formation was scored. Marcus and Sekellick determined that infection of a single virus particle was sufficient to kill the host cell.

The CK ability of VSV was analyzed as a function of U.V.-irradiation administered to the infecting virus. U.V. light will produce uridine dimers in the RNA genome at random points which will prevent transcription of viral messages and therefore expression of viral products downstream of that point. Similar U.V. inactivation experiments (Ball and White, 1976) were used to determine the gene order of VSV. The production of infectious progeny virus naturally requires expression of all gene products. If CK also requires expression of all viral genes, then these two properties should be equally sensitive to irradiation. CK, however, was determined to be 5 times more resistant to U.V.-irradiation indicating that expression of the entire genome is not required to kill the host cell. This result left open the possibility that the infecting VS virion itself is cytotoxic and that cell death does not involve viral expression. To test this possibility, DI particles which contain all 5 viral proteins were extensively purified from wt virus and infected into vero cells in culture. At multiplicities of infection (MOI) up to 70, the particles did not induce CK suggesting that viral expression must occur. Later experiments by McSharry and Choppin (1978) did show that treatment of baby hamster kidney (BHK) cells with purified viral G protein rapidly inhibited host DNA and RNA synthesis but not protein synthesis. In-

hibition required the equivalent amount of G protein per cell as would be found at a MOI of 20,000 to 40,000. Although this number may seem very large, it must be remembered that a great deal of G protein is synthesized in the infected cell. The effect of G protein on CK is not significant at a MOI of 10, however, its presence in large amounts later in infection is certainly detrimental to the cell.

It appeared from U.V. data that viral expression is required for CK. Additional proof came from the use of temperature sensitive (ts) mutants of VSV (Marcus and Sekellick, 1975). Certain mutants, ts for transcription, were not able to induce CK at the nonpermissive temperature. These transcription minus mutants mapped to either the N, NS or L genes reflecting the requirement of all three for viral transcription. Other ts mutants that retained transcription activity but were infectious-virus-negative retained their CK capacity supporting the notion that viral transcription is required for CK.

Marcus and Sekellick (1976) proposed that viral transcription may be required to stimulate interferon production. VSV is an efficient interferon stimulator, and it may be that interferon is responsible for CK. To test this, they used the DI particle, DI-011. DI-011 originally characterized by Lazzarini et al. (1975) is believed to form double-stranded RNA within the infected cell which would be a good inducer of interferon. These particles, however, did not induce CK. Moderate doses of interferon before infection with wt VSV did not prevent CK, but large doses did (Marcus and Sekellick, 1976). The ability of interferon to inhibit protein synthesis and stop the cellular processes necessary for viral transcription and replication before infection began would explain this result. In any case, these

authors unequivocally demonstrated the requirement for VSV transcription in CK which laid the groundwork for future studies.

There are variations in the susceptibility of different cell types to VSV, and also CK by VSV. Robertson and Wagner (1981) ranked the susceptibility of a number of cell types to VSV inhibition of RNA synthesis as 18-81>MPC-11>L cells>HeLa cells. The difference in susceptibility to inhibition did not relate to the specific proteins produced by these cells or to variations in viral gene products. Cellular factors must be involved in the mechanism of CK, but it is not clear if the difference in inhibition relates to one common mechanism of CK or if they reflect the difference in susceptibility to infection.

Inhibition of Macromolecular Synthesis

Death of VSV infected cells is preceded by the rapid inhibition of cellular macromolecular synthesis (Wagner et al., 1984). Presumably, this inhibition is the cause of the host cell's ultimate destruction, but no direct cause and effect relationship has been shown.

McAllister and Wagner (1976) were the first to examine the inhibition of specific cellular protein synthesis in VSV infected L-cells. They found that cellular protein synthesis was 80% inhibited at 5 hours post-infection (p.i.) with a MOI of 10. Investigations using BHK cells, vero cells, HeLa cells and others at a range of multiplicities reported very similar results. Lodish and Porter (1980) examined the inhibition of ten major host proteins by VSV. They determined that host mRNA associated with fewer and fewer ribosomes between 2.5 and 4.5 hours p.i. These mRNAs were not degraded or inactivated. Further studies (Lodish and Porter, 1981) compared the build-up of viral mRNA with the degree of host protein synthesis inhibition. Cell-lines where

inhibition of translation was relatively slow had a slower build-up of viral messages. These authors proposed that inhibition was due to competition of messages for a limited number of ribosomes. As the number of viral messages increased, their possibility of being translated became greater. Jaye et al. (1980), however, in studies of VSV infected cells, determined that host mRNAs were underutilized. They observed apparently normal 80S ribosome complexes in VSV-infected cells and proposed that inhibition of translation was an active viral function and not a consequence of competition. It must be remembered, however, that VSV inhibits host RNA synthesis, and therefore, assembly of new ribosomes which will further indirectly prevent protein synthesis.

Centrella and Lucas-Lenard (1982) used a different approach to examine the regulation of protein synthesis in VSV infected L-929 cells. They prepared translationally active extracts from uninfected and VSV infected cells. Only VSV messages could be translated in extracts prepared from VSV infected cells at 4 hours p.i. Cellular messages were translated if these extracts were supplemented with partially purified eukaryotic eIF-2. They concluded that eIF-2 is somehow altered or inactivated in infected cells. Under these conditions, VSV messages were better able than host messages to be translated. Thomas and Wagner (1983) performed similar experiments with VSV infected L-cells. They found that extracts prepared from VSV infected cells would only translate host mRNA after addition of purified eIF-3 and eIF-4B. Due to the close functional association of these translation factors, it may have been possible that the crude preparation of eIF-2 used by Centrella and Lucas-Lenard contained eIF-3 and eIF-4B activities also.

The viral agent responsible for the inhibition of translation has been more elusive. VSV mutants (McAllister and Wagner, 1976), ts for transcription (L protein) and U.V.-irradiated VSV (Baxt and Bablanian, 1976) were unable to inhibit host translation. More careful studies of U.V.-irradiated VSV determined that only transcription through the N gene was required to shut-off host protein synthesis. With increased U.V.-irradiation preventing synthesis of RNA greater than 200 n.t., there remained a residual inhibitory activity which presumably correlated with transcription of VSV leader RNA. Extremely high doses of U.V.-irradiation to prevent all transcription or heating of the infecting virion to 50°C to inactivate the RNA dependent RNA polymerase overcame shut-off. In summary, it can be said that inhibition of host cell protein synthesis requires primary transcription of the infecting virus up to and including the N gene. If N mRNA synthesis is eliminated, a residual inhibitor activity remains which is associated with leader RNA.

Early reports (Cantell et al., 1962; Wagner et al. 1963; Huang and Wagner, 1965; Yaoi et al., 1970; Weck and Wagner, 1978) determined that cellular RNA synthesis is inhibited in VSV infected cells. Weck and Wagner (1979) analyzed the ability of VSV to inhibit cellular transcription with DI particle and ts mutants. DI particles which contained only sequence information from the 5' half of the genome at MOIs up to 10,000, and ts mutants defective in transcription at the nonpermissive temperature were unable to produce inhibition of RNA synthesis in MPC-11 cells. DI particles containing sequences from the 3' half of the genome were able to inhibit transcription. These results implied that, as was observed with studies on the inhibition of

translation, transcription of a portion of the 3' end of the wt genome was required to induce inhibition of transcription. Again, U.V.-irradiated virus was used in an attempt to identify the viral product responsible. Huang and Wagner (1965) reported that U.V.-irradiated (50,000 ergs/mm^2) VSV at MOIs of 0.5, 5 and 50 did not prevent the inhibition of cellular transcription. Weck et al. (1979) observed that VSV sufficiently irradiated (150,000 ergs/mm^2) to prevent detection of any viral messages did not overcome inhibition. This observation led them to propose that leader RNA may be responsible for host cell transcription inhibition. McGowan and Wagner (1981) and later Grinnell and Wagner (1983) using highly purified VS virions [serotype Indiana (IND) and New Jersey (NJ), respectively] confirmed the observations of Weck et al. (1979) and determined the target size of the transcript required to produce inhibition to be between 50 and 150 n.t.

Dunigan and Lucas-Lenard (1983) performed experiments similar to Weck et al. (1979) and determined that inhibition of transcription was biphasic and that there were two targets, one 42 n.t. (presumably leader RNA) and the other 373 n.t. (presumably N mRNA). Both laboratories identified leader RNA as one viral transcript that is required for inhibition to occur. Transcription of N mRNA was previously found to be required for inhibition of host translation. RNA synthesis requires host proteins so if N message or protein is responsible for inhibition of translation, N would also indirectly inhibit transcription.

McGowan et al. (1982) directly examined the ability of leader RNA to inhibit transcription in vitro. HeLa cell extracts were prepared that were capable of transcribing RNA polymerase II and III genet-

ic elements, the SV40 late promoter and the adenovirus VA genes, respectively. Only the addition of wt leader RNA inhibited transcription of these genes. The DI small RNA product, oligo(dT) selected VSV RNA, and yeast RNA had no effects. Continuing studies (Grinnell and Wagner, 1983) compared VSV leader RNA isolated from serotype IND and NJ and found an exact correlation of their in vitro with their in vivo ability to inhibit transcription. Additionally, they determined that the NJ leader RNA, the better inhibitor of transcription of the two, was present in greater numbers within infected cells. Indiana leader RNA was present in infected cells at up to 550 copies per cell while the NJ leader RNA was present at up to 2900 copies per cell. The conclusion that has been drawn from these reports is that VSV leader RNA is the viral agent responsible for the inhibition of transcription in vitro and possibly in vivo.

A recent article (Dunigan et al., 1986) has challenged this conclusion. The authors compared the build-up of leader RNA with the inhibition of host cellular RNA and protein synthesis in L-cells infected with either wt VSV or a revertant of a ts VSV mutant. They found that VSV leader RNA accumulated to the same extent in both populations of infected cells, but host shut-off was delayed 2 to 3 hours in cells infected with the VSV mutant. Their results, however, do not determine if leader RNA is altered in the mutant virus which would account for the delay in host cell shut-off.

Recent studies of Grinnell and Wagner (1985) compared the ability of synthetic oligodeoxynucleotides, analogous to the sequence of portions of leader RNA to inhibit in vitro transcription. Their results indicated that the AU-rich central region of leader RNA from

nucleotide position 18 to 24 is responsible for inhibition and that the presence of additional flanking sequences enhance this ability. They identified a 65-Kd protein in HeLa cell extracts that reversed the inhibitory action of leader RNA in vitro. The synthetic oligodeoxynucleotides representing the central region of leader RNA were able to bind to a gradient fraction which contained the 65-Kd protein. As yet, the function of this protein and definitive proof that it is a cellular target of VSV leader RNA has yet to be determined.

Yaoi et al. (1970) were the first to examine VSV's affects on cellular DNA synthesis. They observed in VSV_{NJ} infected chick embryo cells that host cell nucleic acid synthesis was rapidly inhibited. Inhibition also occurred using U.V.-irradiated VSV in the absence of detectable viral RNA synthesis or evidence of degradation of cellular nucleic acids. The authors concluded that inhibition was due to a U.V.-resistant component of the infecting VS virion and that viral translation was not required. It wasn't until 1981 that the ability of VSV to inhibit cellular DNA synthesis was reexamined.

McGowan and Wagner (1981) infected exponentially growing mouse myeloma MPC-11 cells and L-cells with VSV. Cells were induced into S phase by thymidine and hydroxyurea treatment before infection producing 90% synchrony of cell cycle in the population. There were no significant differences in the inhibition of DNA synthesis produced by VSV between synchronized or unsynchronized MPC-11 or L-cells. Additionally, they found that viral infection had no affect on thymidine uptake, DNA degradation, in vitro thymidine kinase activity or in vitro DNA polymerase activity. As was observed in studies of the inhibition of cellular RNA synthesis (Weck and Wagner, 1979), VSV ts mutants, tran-

scription minus at the nonpermissive temperature and DI particles derived from the 5' end of the standard VSV genome did not inhibit host cell DNA synthesis, again indicating that transcription is required for inhibition. U.V. targeting determined the size of the transcript responsible for the inhibition of DNA synthesis, and this corresponded with the U.V. target size reported for the inhibition of host RNA synthesis (Weck et al., 1979; Grinnell and Wagner, 1983). These results suggested that the viral agent responsible for the shut-off of both cellular DNA and RNA synthesis in VSV-infected cells was leader RNA.

Therefore, it seemed possible that the inhibition of DNA synthesis was a consequence of the inhibition of RNA synthesis. Eukaryotic replication requires RNA synthesis to produce a primer for the initiation of DNA synthesis. McGowan and Wagner (1981) using VSV infected MPC-11 cells labeled with ^{3}H-thymidine, ^{3}H-uridine or ^{3}H-amino acids observed that the rates of inhibition of DNA or RNA synthesis were nearly identical. Their data implied that VSV directly inhibits both DNA and RNA synthesis or a parameter common to each.

The use of an in vitro transcription assay to examine the affects of VSV transcription products on the inhibition of RNA synthesis has identified leader RNA as the most likely viral agent responsible for inhibition. Systems to study cellular replication in vitro are not available, however, much is known about the replication of adenovirus DNA in vitro and in vivo. Using the adenovirus in vitro replication assay, it may be possible to determine the affects of VSV and VSV leader RNA on eukaryotic DNA synthesis.

Adenovirus Structure and Life Cycle

Adenovirus (from Greek adenos = gland) is a small, double-stranded DNA virus first discovered in 1953 as the causative agent of upper respiratory tract infections in children of the Washington, D.C. area (Rowe et al., 1953). Forty-one different human species (formerly referred to as serotypes) under the genus, mastadenoviridae (from Greek mastos = breast or mammalian) have been identified which produce a variety of ailments including gastrointestinal diseases, urinary tract infections, acute and chronic respiratory diseases or latent infections with no overt clinical symptoms (Straus, 1984). Many species are oncogenic in rodents, and all are able to transform rodent cells in culture. The prototypical adenoviruses upon which the majority of research has been done are adenovirus serotype 2 (Ad2) and adenovirus serotype 5 (Ad5), both members of the nononcogenic group C adenoviruses (Huebner, 1967).

Ad2 is a nonenveloped, isometric virus with icosohedral capsids consisting of 252 capsomers, 240 hexons and 12 pentons each non-covalently associated with a fiber protein (Nermut, 1984). Infection begins by attachment of the viral fiber protein to the cell receptor. Svensson et al. (1981) have identified a 40 to 42-Kd glycoprotein of HeLa cells as the Ad2 receptor which is expressed at roughly 10^5 copies per cell. The viral nucleocapsid enters the cell and is transported to the nucleus where early transcription begins.

The Ad2 genome is 35,937±9 base pairs (bp) in length (Roberts et al., 1986). Variation in reported genome length is the result of ambiguities in the strains of stock carried in different laboratories. Around 50 polypeptides are encoded within the Ad2 genome whose expres-

sion is divided into two distinct phases (Akusjarri et al., 1986). Polypeptides expressed prior to viral DNA replication are classified as early phase products. These early polypeptides comprise all the viral proteins necessary for replication and include the viral DNA polymerase (140-Kd), the DNA binding proteins (72-Kd), the precursor of the terminal binding-protein (pTP) (87-Kd) and at least 22 additional proteins whose functions are currently under investigation. All products expressed after the onset of DNA replication are called late phase products. This group of polypeptides predominantly includes the viral structural proteins (Esche, 1986). Complete adenovirus particles form as crystalline arrays within the infected cell nucleus which are only released after mechanical or chemical disruption of the cell membrane.

Adenovirus Replication

Eukaryotic and adenovirus DNA synthesis are semi-conservative processes (Ginsberg, 1980; Tamanoi, 1986). Each strand is replicated by a polypeptide complex consisting of a polymerase, a primase and a number of associated factors. Typically, eukaryotic replication is initiated by small RNA molecules laid down by DNA primase which are eventually excised and replaced by DNA. Adenovirus replication does not require RNA synthesis but binding of a viral protein for primer formation (Tamanoi, 1986). This method of protein primed replication is also found to occur in a small number of phages which infect Bacillus subtilis (Kawamura and Ito, 1977). Adenovirus replication is one of the best understood models of eukaryotic replication. Most of what has been discovered was through the use of the adenovirus in vitro replication system developed by Challberg and Kelly (1979b) which is believed to mirror in vivo replication. The following is a summary of what has

Figure 4. <u>Ad2 Semi-conservative DNA Replication</u>. Ad2 DNA replication initiates at each 5' end of the genome by the covalent attachment of dCMP to the precursor of the viral terminal protein (pTP) in association with the viral polymerase (Adpol) and nuclear factor I (NFI). Ad2 DNA polymerase then synthesizes complementary DNA from the pTP-dCMP primer while continually displacing the opposite strand of the genome which is coated with the viral DNA binding protein (-●-●-●-). Nuclear factor II (NFII), a type I topisomerase, assists polymerization by unwinding the genome during elongation. The viral terminal protein (TP) is present on mature Ad2 genome and is required for the initiation of Ad2 DNA replication.

ADENOVIRUS REPLICATION
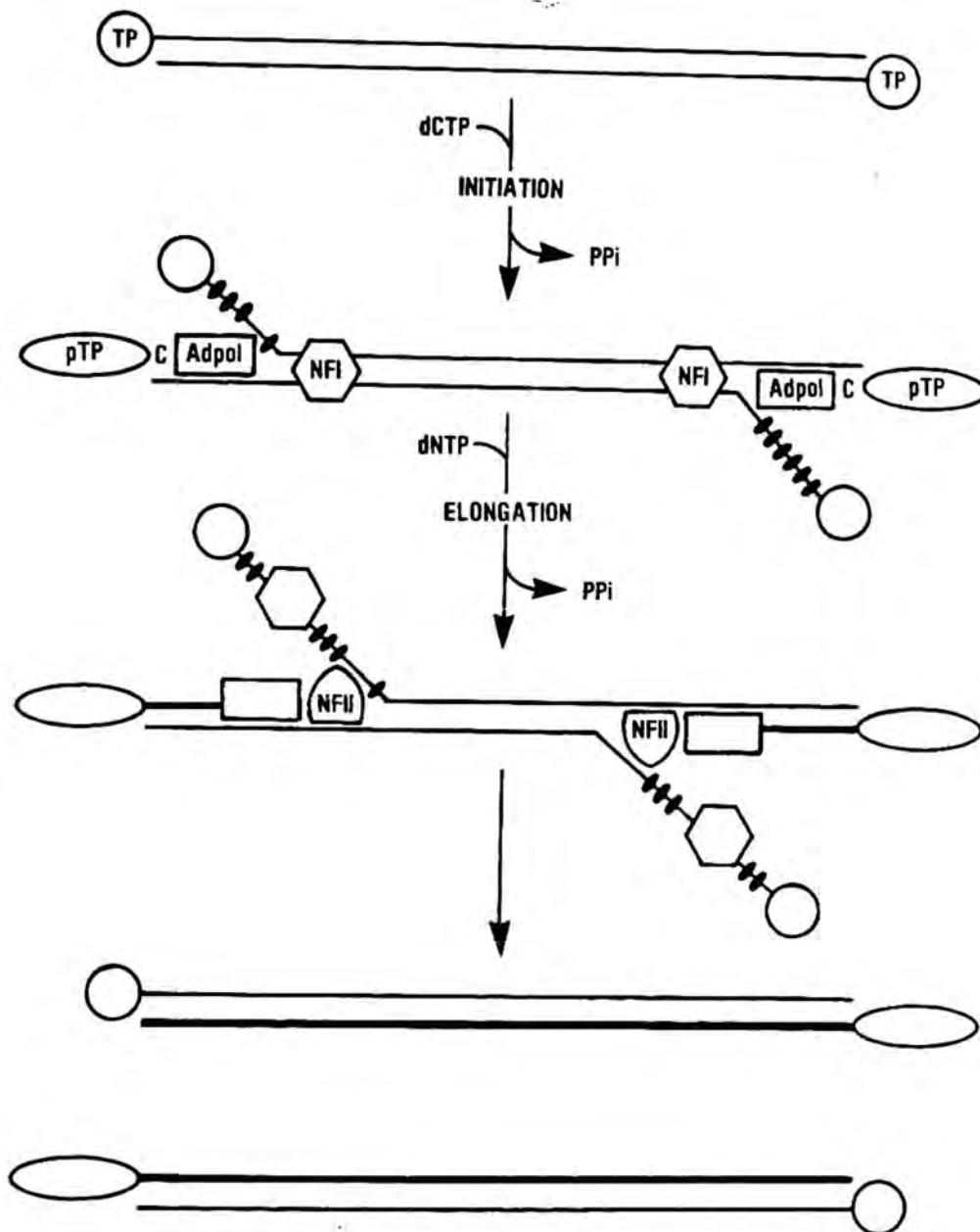
been learned of the process of adenovirus replication in vitro as it applies to this dissertation.

Adenovirus replication is initiated at each terminus of the linear DNA genome (Figure 4). Briefly, initiation begins by the co- valent attachment of the pTP with dCTP in the presence of viral tem- plate creating a phosphodiester linkage between the hydroxyl group of a serine residue of the pTP and the 5' phosphate group of dCMP (Lichy et al., 1981). This places a free 3' hydroxyl of the dCMP at the terminus of the genome which is necessary for the viral DNA polymerase to begin DNA synthesis. Initiation also requires a host cell derived protein referred to as nuclear factor I (NFI). NFI is a DNA binding protein with no ATPase, DNA polymerase, RNA polymerase or nuclease activities but is required for the initiation of replication (Nagata et al., 1982). The size of NFI was originally reported as 47-Kd and was eluted as one peak from a DNA-cellulose column in 0.25 M NaCl. SDS-poly- acrylamide gel electrophoresis did not resolve NFI as a single band but as multiple bands ranging in size from 40-Kd to 65-Kd (Philip J. Rosen- feld, personal communication). Investigators are presently trying to resolve this discrepancy. Gronostajski et al. (1984) identified human DNA sequences which bind to NFI in vitro. They determined that one NFI binding site exists every 100-Kbp, the spacings of the origins of replication of many human cell-line (Lewin, 1985) and proposed that NFI may function in vivo during the initiation of replication, transcrip- tion or recombination. A second host cell protein, NFII, required for the elongation of already initiated DNA chains is a type I topoiso- merase and is important for unwinding the adenovirus template (Nagata et al., 1983). In the absence of NFII, initiation will occur, but only

about 25% of the genome will be synthesized. The addition of both of these factors which were isolated from the HeLa cell nuclear extracts to the adenovirus replication reaction in vitro stimulates DNA synthesis 15-fold. A third viral protein, the 72-Kd DBP is required for replication of the full-length Ad2 genome. Its plenary of functions are not known, but it is thought to be involved with elongation (Kaplan et al., 1979). All five proteins are necessary for the synthesis of full-length genomic DNA.

In addition to the five required proteins, two additional elements may affect adenovirus replication. The first is a host cellular protein that will substitute for the viral DNA binding protein. It was observed that crude extracts prepared from cells infected with Ad5 ts 125 (ts for the viral DNA binding protein) can support replication in vitro at the nonpermissive temperature which suggests that the DNA binding protein is not required for initiation. However, when replication is reconstructed using purified proteins, the DNA binding protein is absolutely required. These two results could imply that a host cellular protein is substituting for the viral DNA binding protein (Friefeld et al., 1983). It was reported by van der Vliet et al. (1984) that a second element, an RNA fraction extracted from HeLa cells, would stimulate Ad2 replication in vitro. While the minimal requirements for adenovirus replication in vitro have been defined, it is likely that there are additional proteins or factors which affect or regulate adenovirus replication in vivo.

The ends of the Ad2 genome, the viral origins of replication, are each bound at their 5' terminus with a protein (pTP) required for the initiation of replication. These sequences contain an inverted

terminal repeat (ITR) of 102-bp which can be separated into two distinct regions, an AT-rich region from n.t. 1 to 54 and a GC-rich region from n.t. 55 to 102. The sequences of the AT-rich region are highly conserved among human adenoviruses and play an important part in the initiation of viral replication. The GC-rich region is not as highly conserved, but there are blocks of conserved sequences whose functions are as yet unknown.

Tamanoi and Stillman (1982) clearly demonstrated that the ITR region is the viral origin of replication. A plasmid containing 33-kb of sequence derived from the Ad5 left terminus was cleaved with the restriction endonuclease, EcoRI. Linearization by EcoRI created a terminal sequence which corresponds to the terminal sequence of the Ad5 genome. Linearization using another restriction enzyme, PstI, put the Ad5 terminal sequences 20-bp internal to the plasmid end. Only when the Ad5 terminal sequences were at the exact end of the DNA, however, was the plasmid replicated in vitro. The presence of 20-bp or more separating the Ad5 sequences from the end of the DNA molecule prevented pTP from binding and initiating DNA synthesis. Of great surprise was the finding that a supercoiled plasmid, which contains the left and right terminal sequence of Ad5 separated by a kanamycin resistance gene, transfected into 293 cells, could be replicated in vivo (Hay et al., 1984). Each Ad5 sequence was replicated exactly forming short linear DNA segments within the cell. In vivo replication of this plasmid was found to be dependent on the host cell protein, pL (Guggenheimer et al., 1984). pL has no affect on adenovirus replication in vitro and will not substitute for any of the five required proteins. pL has been identified as a exonuclease, and its function in the host

cell is currently under investigation (Mark Kenny, personal communication).

Within the ITR AT-rich region are two domains which play important roles in the initiation of Ad2 replication. Domain I is the region between the 9th- and 18th-bp and is perfectly conserved among human adenoviruses. Point mutations created in this region significantly decrease replication activity of the template (Tamanoi and Stillman, 1983; Challberg and Rawlins, 1984). Point mutations just outside the sequence did not affect template activity to the same extent. It was concluded that domain I is absolutely required for the pTP-DNA polymerase-template complex to form and is the recognition sequence for the Ad DNA polymerase. Domain II is the NFI binding site as determined by DNase I footprinting experiments (Nagata et al., 1983) and filter binding experiments (Guggenheimer et al., 1984) using point mutations which defined the region between the 15th and 48th-bp.

Adenovirus replication in vitro is an excellent system to study the inhibition of DNA synthesis by VSV. First, adenovirus replication is well understood. Many of the required proteins (both cellular and viral) have been identified and characterized. Second, DNA synthesis in vitro is believed to mimic replication in vivo during initiation and completion of synthesis of the full-length template. Third, adenovirus replication uses host cell machinery and proteins. Fourth, adenovirus replication does not require concomitant RNA synthesis for primer formation. Replication is initiated through binding of the pTP to the origins of replication on the template. Therefore, the effects of VSV on DNA synthesis can be determined directly and not indirectly as a consequence of the inhibition of RNA synthesis. Fifth, preliminary

experiments have shown that upon co-infection of Ad2-infected cells with VSV, Ad2 protein synthesis is inhibited and replication prevented.

Results presented in this dissertation indicate that the small (47 n.t.) leader RNA of VSV will inhibit Ad2 replication in vitro using crude HeLa cell nuclear extracts and purified protein fractions. No other RNA tested would inhibit specific Ad2 DNA synthesis nor would the small (46 n.t.) RNA transcribed from VSV defective interfering particles even when added at 10 times the amount required for leader RNA to produce inhibition. Small synthetic oligodeoxynucleotides analogous to the sequence of short regions of VSV leader RNA indicate that the central A-rich region from n.t. 8 to 29 is important for the inhibition of Ad2 DNA replication in vitro. The significance of this region of leader RNA is discussed in relation to other salient viral and eukaryotic sequences.

Data from sucrose gradients and nondenaturing polyacrylamide gels have shown that J8, a synthetic oligodeoxynucleotide analogous to the sequence of leader RNA, associates with a protein present in the Ad2 infected and uninfected HeLa cell nuclear extracts. U.V.-irradiation of replication mixtures containing J8 has crosslinked this synthetic with at least one protein within HeLa cell extracts of approximately 137-Kd. Reconstitution experiments using HeLa cell nuclear fractions isolated from gradients and gels have been unsuccessful; however, inhibition can be overcome with the addition of supplemental Ad2-infected or uninfected HeLa cell nuclear extract.

Further experiments have demonstrated that J8 prevents the initiation step of Ad2 replication by preventing pTP-dCMP complex formation which requires the Ad2 DNA polymerase, the Ad2 pTP and NFI. Nonspecific DNA polymerase assays have shown that J8 will inhibit DNA

synthesis by both the Ad2 DNA polymerase and HeLa cell DNA polymerase enzymes. The addition of either of these two proteins to _in vitro_ replication reactions will restore Ad2 specific DNA synthesis which had been inhibited by leader suggesting that the viral DNA polymerase is the target of leader RNA in these _in vitro_ assays. The relationship of these results to the process of VSV infection _in vivo_ is discussed.

Materials and Methods

Growth of Cells. BHK-21 cells, a gift from Dr. Robert R. Wagner of the University of Virginia, Charlottesville, Virginia, were grown as monolayers at 37°C in minimal essential media plus Earle's salts (EMEM) supplemented with 5% fetal calf serum (FCS). HeLa cells, a gift from Norman Cooper and Dr. Bernard Moss of the National Institutes of Health, Bethesda, Maryland, were maintained as monolayers in 5% FCS/EMEM or as spinner cultures grown in minimal essential media for suspension cultures (SMEM) supplemented with 5% horse serum (HS) at 37°C. Monolayers were passed in 150 cm^2 plastic or glass flasks which reached confluency in 3 days. Spinner cultures were maintained between 2 and 8 x 10^5 cells/ml and fed daily.

Growth and Preparation of Viruses. Wild-type VSV serotype Indiana and defective interfering particle type 011 (DI-011), gifts from Dr. Robert R. Wagner of the University of Virginia, Charlottesville, Virginia, were grown in HeLa or BHK-21 cells from stock which had been plaque purified from L-cell monolayers (Emerson, 1976). Cells were infected with VSV or VSV plus DI-011 at a MOI of 0.1 in a small volume, absorbed for 1 hour and incubated with fresh media supplemented with 2.5% serum for an additional 16 to 19 hours. Cell debris was removed by centrifugation at 900 xg for 20 minutes at 4°C. All subsequent steps were performed at 4°C or on ice. The supernatant fluid was centrifuged at 80,000 xg for 90 minutes through a 2 ml pad of 50% glycerol in phosphate buffered saline (PBS; 0.85% NaCl, 2.6 mM KCl, 8 mM Na$_2$ HPO$_4$, 1.5 mM KH$_2$ PO$_4$). The resulting pellet was resuspended in PBS and layered over a 0% to 40% continuous sucrose gradient containing 50 mM Tris, 0.25 M NaCl, 0.5 mM EDTA (pH 7.6) and centrifuged for 90 minutes at

35,000 xg. The band of VSV or DI-011 was harvested, diluted 5-fold with PBS and pelleted through a 2 ml pad of 50% glycerol in PBS at 60,000 xg for 90 minutes. The virus pellet was resuspended in PBS, overlayed on a 0% to 40% potassium tartrate gradient in PBS, centrifuged for 18 hours at 35,000 xg, the visible band harvested, diluted and pelleted as before. The virus pellet was drained, washed with PBS and suspended in PBS at a concentration of 2 mg/ml (protein) and stored at 4°C for short periods or -80°C for extended times.

Adenovirus serotype 2, a gift from Drs. Christine Lally and Barrie Carter of the National Institutes of Health, Bethesda, Maryland, was grown in HeLa cells. Cells were infected at a MOI of 10, absorbed in a small volume for 1 hour and incubated with fresh media supplemented with 2.5% serum for 40 to 46 hours. Cells were pelleted at 200 xg for 5 minutes, resuspended in 1/50 their original volume of infection media and frozen to -80°C. Virus was released from the cells by three cycles of freezing and thawing, the debris removed by centrifugation at 900 xg for 20 minutes and stored at 4°C for short periods or -80°C for extended times.

Determination of Ad2 and VSV Titers. Ad2 was titered on HeLa cells using a modification of the procedure of Williams (1970). Nearly confluent 35 mm monolayers of cells were infected with a series of dilutions of the stock Ad2 virus. After 1 hour of adsorption in serum-free media, media was removed, and cells were incubated in EMEM/2.5% FCS at 37°C for 24 hours. Incubation media was removed and fresh media plus 0.5% Noble agar was overlayed onto the cells. Incubation was continued. After 3 to 5 days, a second overlay of the same composition plus 0.02% neutral red was added. Twelve to 24 hours

later, plaques were counted. VSV was titered on BHK-21 cells by the method of McGowan and Wagner (1981). Confluent 35 mm monolayers of cells were infected with a series of dilutions of stock VSV. After 1 hour of adsorption in serum-free media, media was removed, and the cells were incubated in EMEM/2.5% FCS for 24 to 48 hours. Incubation medium was removed, cells were stained with 0.02% trypan blue, and plaques were counted.

Ad2-VSV Coinfections. Ad2 was infected at a MOI of 10 to confluent HeLa cell monolayers in 35 mm dishes as described. At various times after infection (1 hour to 12 hours), VSV serotype Indiana was co-infected at a MOI of 10 to these same cells as previously described with the following modifications. After adsorption, cells were fed with methionine-free EMEM supplemented with 2.5% FCS plus 100 μCi of ^{35}S-methionine (specific activity of 800 Ci/mM). Cells were incubated for an additional 4 hours, pelleted at 900 xg for 5 minutes, washed with PBS and lysed in disruption buffer (50 mM Tris pH 7.0, 4% sucrose, 2% sodium dodecylsulfate (SDS), 5% 2-mercaptoethanol, 50 μg/ml bromo-phenol blue (BPB). Lysates were sonicated for 60 seconds, heated at 100°C for 60 seconds and applied to 12% SDS-polyacrylamide gels for electrophoresis.

In Vitro Transcription of Vesicular Stomatitis Virus and Isolation of Wild-type and DI-011 Leader RNA. Vesicular stomatitis virus was tran-scribed in vitro in the presence of radiolabeled ^{32}P-UTP (specific activity of 3000 Ci/mM) (McGowan et al., 1982). Two mg of purified VSV were incubated at room temperature in transcription buffer [10 mM Hepes pH 7.5, 140 mM NaCl, 7.5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 0.2%

Triton X-100]. After 15 minutes, UTP (or 50 μCi of α^{32}P-UTP), CTP and GTP were added to final concentrations of 2.5 mM and ATP to 4 mM. Incubation was continued at 31°C for 4 hours. The reaction mixture was then centrifuged at 285,000 xg for 50 minutes at 15°C and extracted twice, first with an equal volume of phenol-chloroform (1:1) and second with an equal volume of chloroform. RNA was precipitated from the solution with the addition of 0.3 M NaOAc pH 4.8, 2-1/2 volume of 95% ethanol and incubation at -20°C for 24 hours. RNA was pelleted at 16,000 xg for 20 minutes and resuspended in water for studies requiring total VSV RNA or in 8 M urea, 0.01% BPB, 0.01% xylene cylanol (XC) for electrophoresis into 20% acrylamide, 8 M urea gels to isolate leader RNA. Upon autoradiography of the gel, the position of leader RNA could be determined. The gel slice containing leader RNA was removed and the RNA eluted by incubation at 37°C in elution buffer [500 mM NH$_4$OAc, 10 mM Mg$_2$OAc, 0.5% SDS, 1 mM Na$_2$EDTA] for 24 hours. The eluate was passed through a 0.22 micron Millipore filter, ethanol precipitated as before, resuspended in sterile water and quantitated spectrophotometrically.

Preparation of Ad2-infected and Uninfected HeLa cell Extracts. Replication extracts were prepared as reported by Challberg and Kelly (1979b) to final protein concentrations of 15 to 20 mg/ml. Uninfected extract was prepared in the same manner as infected without the addition of virus. One liter of HeLa cells at 3 to 5 x 10^5 cells/ml were infected at a MOI of 10 in a small volume of SMEM. After 1 hour of adsorption, fresh SMEM was added up to the original volume, supplemented with 5% HS and incubation was continued at 37°C. At 2 hours p.i., 10 ml of 1 M hydroxyurea were added. At 21 hours p.i., cells

were pelleted at 900 xg for 5 minutes and kept at 4°C or on ice for all subsequent steps. The cell were resuspended in 10 ml of cold buffer A (0.22 M sucrose, 20 mM Hepes pH 7.5, 5 mM KCl, 2 mM DTT), centrifuged at 1500 xg for 3 minutes, resuspended in 5 ml of buffer A without sucrose, incubated on ice for 10 minutes, lysed in a dounce homogenizer 10 times and centrifuged at 650 xg for 2 minutes. The supernatant was removed and stored at -80°C as cytoplasmic extract. The pellet was resuspended in 2-1/2 ml of 10% sucrose/50 mM hepes pH 7.5 and frozen to -80°C. The frozen suspension was thawed on ice, NaCl added to a 0.2 M final concentration, incubated for 1 hour on ice and centrifuged at 16,000 xg for 5 minutes. Supernatant was removed and stored as nuclear extract at -80°C. Protein concentration was determined according to the method of Lowry et al. (1951).

Preparation of the Ad2 Terminal Protein - DNA Template. Ad2 template was isolated and purified as described by Sharp et al. (1976). Ad2 stock from 1 liter of HeLa cells was centrifuged into 5 ml pads of cesium chloride/25 mM Hepes pH 7.5 at a density of 1.45 g/ml at 110,000 xg for 50 minutes. The visible band of virus was removed, the density adjusted to 1.34 g/ml with cesium chloride and centrifuged at 285,000 xg for 24 hours. The virus band was removed and dialyzed against PBS to remove the cesium chloride. An equal volume of 8 M guanidine HCl was added to the virus solution on ice, incubated for 5 minutes, overlayed onto a 5, 12.5, 20% sucrose step gradient containing 4 M guanidine HCl and centrifuged for 21 hours at 210,000 xg. The gradient was fractionated and the fraction quantitated spectrophotometrically. The fractions containing significant quantities of DNA were pooled, dialyzed against 10 mM Tris pH8, 1 mM EDTA, 1 mM phenyl-

methylsulfonyl fluoride (PMSF) and stored at 4°C at 35 to 50 ng/μl (DNA).

HindIII Cleavage of Ad2 Template. Ad2 template was purified as described and cleaved in 50 μl batches with restriction enzyme. Forty-three μl of purified Ad2 template was incubated in digestion buffer (0.05 M NaCl, 10 mM Tris pH 7.4, 10 mM MgCl$_2$) and 2 μl of the restriction endonuclease HindIII (100,000 units/ml) at 37°C for 30 minutes. Cleaved template was stored at 4°C until use.

Adenovirus In Vitro DNA Replication. In vitro replication was carried out as described by Challberg and Kelly (1979a). Briefly, the replication extract was incubated at 31°C for 60 minutes with 35 to 50 ng (75-100 pM) of HindIII cleaved (except where noted) Ad2 template, replication buffer (25 mM Hepes pH 7.5, 5 mM MgCl$_2$, 1 mM DTT, 3 mM ATP), 50 uM each of the deoxynucleoside triphosphates: dATP, dGTP, and dTTP, 10 μCi of ^{32}P-dCTP (specific activity of 3000 Ci/mM) and exogenous nucleic acid as specified. The mixture was digested with 100 μg of proteinase K with 1% SDS for 120 minutes and ethanol precipitated as described previously. The pellet was resuspended in a small volume of 5% glycerol, 0.01% BPB and electrophoresed into a 0.8% agarose gel. The gel was dried on a vacuum dryer without heating and autoradiographed. The amount of replication which occurred was quantitated two ways. First, the autoradiograph was scanned with a Ortec densitometer, and the peaks produced on graph paper by the two terminal Ad2 HindIII fragments quantitated by weighing. Second, the two terminal Ad2 HindIII fragments were removed from the gel and Cerenkov counted. The results from each method were averaged and are presented in tabular form.

Synthetic Oligodeoxynucleotides. Synthetic oligodeoxynucleotides J1, J2, J5, J7, J8 and J10 were obtained from Dr. Gerald Zon of the National Institutes of Health, Bethesda, Maryland. Each was HPLC purified and sequenced according to the modified version of the Maxam and Gilbert (1980) sequencing technique (Ruslow, 1983). Briefly, synthetic oligo-deoxynucleotides were 5' end labeled with ^{32}P and the reaction mixtures diluted with water to about 20,000 cpm/μl. Typical Maxam and Gilbert chemical cleavage reactions were performed with the following modifications. Nucleotide specific reactions were supplemented with 1 μg of calf thymus DNA; 50 μg of deproteinized tRNA was used as carrier for ethanol precipitation and incubation times were reduced 50%. After reactions were completed, samples were suspended in 0.01% BPB, 0.01% XC, 8 M urea and electrophoresed into 20% acrylamide, 8 M urea sequencing gels for autoradiography. Poly dI/dC ($S_{w,20}$ 6-9) was a gift from Dr. Anna Riegel of the National Institutes of Health, Bethesda, Maryland.

Radiolabeling of Synthetic Oligodeoxynucleotides. Synthetic oligode-oxynucleotides were ^{32}P-labeled at their 5' termini. One μg of synthetic DNA was incubated with 50 μCi of γ ^{32}P-ATP (specific activity 3,000 Ci/mM) and 1 unit of T4 polynucleotide kinase in kinase buffer (50 mM Tris pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA) for 60 minutes at 37°C. Labeled molecules were purified by precipitation with 0.3 M NaOAc (pH 4.8) and 2.5 volume 95% ethanol, centrifugal dialysis through Sephadex G25 (medium) or electrophoresis into 20% polyacryl-amide 8 M urea gels and elution from the gel as previously described. 5' end-labeled synthetic molecules were determined to be greater than 90% full-length by electrophoresis into 20% acrylamide, 8 M urea gels,

autoradiography and densitometric scans of the resulting bands.

<u>Autoradiography</u>. Radiolabeled gels were exposed to Kodak XAR-5 or XRP-5 X-ray film at -80°C for autoradiography. On occasion, Dupont brand lightening plus intensifying screens were used.

<u>Gel Electrophoresis</u>. Ad2 <u>Hind</u>III DNA fragments were separated by electrophoresis in 0.8% agarose gels using tris-borate-EDTA (50 mM Tris, 40 mM borate, 1 mM EDTA) running buffer. Electrophoresis was performed at 200 volts until the tracking dye was near the end of the gel. Gels were dried on a vacuum dryer at room temperature and autoradiographed.

Eight percent and 12% SDS-polyacrylamide gels were used for protein separation contained an initial 5% stacking gel as described by Laemmli (1970). The gel polymerization reaction was initiated by the addition of TEMED (N, N, N', N'-tetramethylethylenediamine; 50 μl for 100 ml of acrylamide) and ammonium persulfate (0.8 ml of a 10% w/v solution for 100 ml of acrylamide). Bis acrylamide was crosslinked at a ratio of 30:1 (acrylamide:Bis). Electrophoresis was performed in tris-glycine-SDS [200 mM glycine, 25 mM Tris (pH 8.3), 1% SDS] running buffer at 50 volts until the tracking dye cleared the stacker and at 200 volts until the tracking dye was near the bottom of the gel. The gels were dried at 70°C on a vacuum dryer and autoradiographed. Nondenaturing 12% polyacrylamide gels were prepared and run in the same manner as SDS-polyacrylamide gels in the complete absence of SDS, a modification of the procedure by Fried and Crothers (1981). Gels were not dried down after electrophoresis, but sealed in plastic bags and autoradiographed. Twenty percent acrylamide, 8 M urea sequencing gels

were prepared and run as described by Maxam and Gilbert (1980). Electrophoresis was performed in tris-borate-EDTA running buffer at 2000 volts until the tracking dye was near the end of the gel. Gels were covered with saran wrap and autoradiographed.

Silver Staining of SDS-polyacrylamide Gels. After electrophoresis, gels to be silver stained were soaked in 50% methanol for 12 to 24 hours then transferred to the staining solution (0.8% $AgNO_3$, 0.21 M NH_4OH, 19 mM NaOH) and stained for 20 minutes. Gels were washed with water for 15 minutes and the impregnated silver reduced by soaking in developer (0.0005% citric acid, 0.016% formaldehyde) for 20 minutes. Gels were stored in 50% methanol or dried on a vacuum dryer at 70°C after 3 days in 50% methanol.

Electron Microscopy. VSV was negatively stained with phosphotungstic acid according to the procedure of Straus et al. (1981). Parlodion covered copper grids were touched to a solution of VSV in PBS. Grids were blotted to absorbent paper to remove excess fluid, immersed in 2% phosphotungstic acid pH 7.4, blotted dry and rinsed in double distilled water for 10 seconds. Grids were blotted, air dried and examined in a Zeiss EM10A transmission electron microscope.

Hybridization Experiments. Ad2 DNA template was transferred (blotted) according to the procedure described by Woods (1984) onto BA-85 nitrocellulose paper (Schleicher and Schuell, Keene, NH) using an IBI (New Haven, CT) slot blot apparatus. The paper was dried in a vacuum oven at 80°C for 2 hours and prehybridized by incubation in a Dazey brand seal-a-meal baggies containing hybridization solution (90 mM sodium citrate, 900 mM NaCl, 0.05% sodium pyrophosphate, 100 μg/ml

calf thymus DNA, 20 μg/ml tRNA, 0.02% bovine serum albumin, 0.02% ficoll type 400, 0.02% polyvinylpyrrolidone) for 2 hours at 50°C. 10^6 cpm of gel purified J8 or J10 (5' end labeled with ^{32}P as previously described) were added to the baggies and incubation continued for 24 hours. The blots were then removed from the baggies and washed in 6 liters of wash solution (900 mM NaCl, 90 mM sodium citrate, 0.05% sodium pyrophosphate, pH 7.0) at 37°C for 2 to 4 hours and auto-radiographed.

Construction and Elution of AH Sepharose 4B Columns. According to the procedure of Pharmacia (1983), 1 gram of AH Sepharose 4B was swelled in 0.5 M NaCl for 15 minutes at room temperature, washed through a center glass filter with 200 ml of 0.5 M NaCl and 50 ml of H_2O adjusted with HCl to pH 4.5. A solution of 0.1 M carbodiimide (CDI) was made in H_2O adjusted with HCl to pH 4.5 and 10 μl added to the agarose. Thirty μg of 5' end, ^{32}P-labeled J7 (kinased as previously described) was dissolved in H_2O adjusted with HCl to pH 4.5 at 0.3 μg/μl and added to the CDI-Sepharose solution. The pH remained between 5.5 to 5.8 during the course of the reaction. The solution was rotated end over end at room temperature for 24 hours after which excess ligand and CDI were washed away by three washes of 10 ml each of H_2O adjusted with HCl to pH 4.5. The mixture was quenched with 2.5 ml of acetate then washed four times with 20 ml each of 0.3 M acetate buffer pH 4.0, 0.3 M borate buffer pH 8.0, H_2O and replication buffer. The J7 coupled agarose was stored at 4°C until use. Cerenkov counts of fractions of coupled agarose determined that 17% of the 30 μg of J7 had bound to the agarose. Additional agarose was swelled and treated in the manner described above without coupling to J7 and used as a control for certain experiments.

One hundred μl of coupled J7-agarose was loaded onto a column created within a Pasteur pipette. The column was washed with replication buffer and incubated with 0.5 mg/ml bovine serum albumin for 4 hours at 4°C. The column was washed with 15 ml replication buffer. 100 μl of Ad2 infected HeLa cell nuclear extract were added and incubated in the column for 30 minutes at room temperature. The column was washed with 15 ml replication buffer and eluted with 15 ml each of increasing concentration of sodium phosphate buffer pH 7.5 (50 mM, 250 mM, 500 mM, 750 mM, 1 M, 2 M). The first four 200 μl fractions from each elution were collected, dialyzed 3 times against 1 liter of replication buffer with 20% glycerol and stored at 4°C.

Purification of Proteins Required for Ad2 In Vitro DNA Replication and DNA Polymerase Assays. Nuclear factor I was purified (210-fold) by Philip Rosenfeld of The Johns Hopkins Medical School, Baltimore, Maryland according to the procedure of Nagata et al. (1983). The Ad2 protein fractions containing the Ad2 DNA polymerase and the precursor of the Ad2 terminal binding protein and the Ad2 DNA binding protein were purified (>95%) by Dr. Jeffrey Ostrove of the National Institutes of Health, Bethesda, Maryland according to the procedure of Ikeda et al. (1981). HeLa cell DNA polymerase-primase complex was purified (290-fold) by Mark Kenny of the Memorial Sloan-Kettering Cancer Center, New York according to the procedure of Longiaru et al. (1979). Calf thymus DNA polymerase-primase was purified (>99%) by Dr. Andrew M. Holmes of the Uniformed Services University of the Health Sciences, Bethesda, Maryland according to the procedure of Holmes et al. (1986).

Assay for pTP-dCMP Complex Formation. Reaction mixtures of 50 ml each contained: 25 mM Hepes pH 7.5, 5 mM $MgCl_2$, 4 mM DTT, 3 mM ATP, 0.5 mM α ^{32}P-dCTP (specific activity 400 Ci/mM), 10 μg BSA, 10 ng of Ad template, 5 μl of Ad2 infected HeLa cell nuclear extract. After incubation for 1 hour at 31°C, then 5 minutes at 70°C, 25 μl of micrococcal nuclease solution (3 x 10^3 units/ml micrococcal nuclease, 60 mM Tris pH 8.0, 18 mM $CaCl_2$, 5 mM $MgCl_2$, 10% glycerol, 1 mM PMSF) was added. Incubation was continued at 37°C for 30 minutes then 25 μl of 50% trichloroacetate (TCA), 2 μg/ml deoxycholate were added. The mixture was put on ice for 10 minutes, centrifuged at 12,000 xg for 5 minutes, the pellet resuspended in 25 μl of sample buffer (5% glycerol, 0.001% phenol red, 5% SDS, 50 mM Tris pH 6.8, 100 mM 2-mercaptoethanol) and electrophoresed into an 8% SDS-polyacrylamide gel with a 4% stacking gel.

Ad2 In Vitro DNA Replication with Purified Protein Fractions. In vitro replication of Ad2 template was performed as described by Ikeda et al. (1981) with modifications. Replication reactions of 20 μl contained 25 mM Hepes pH 7.5, 4 mM DTT, 3 mM ATP, 5 mM $MgCl_2$, 0.02% BSA, 20 mM NaCl, 40 μM each of dATP, dTTP, dGTP, 4 μM ^{32}P-dCTP (specific activity of 200 cpm/pM), 60 ng Ad2 DNA template, 2.3 units of the Ad2 DNA polymerase-pTP complex, 4.7 units of the Ad2 DBP, 50 ng of NFI and extraneous substrate as specified. Reactions were incubated for 2 hours at 31°C then blotted onto GF/C filter papers with an equal volume of 5 mM ATP, washed with 5% TCA, 1 N HCl, 95% ethanol and Cerenkov counted.

DNA Polymerase Assay. This assay measures the conversion of ^3H-dTTP into acid insoluble product as described by Schlaback et al. (1971).

Reaction mixtures of 62 μl contained 40 mM potassium phosphate pH 7.0, 8.0 mM MgCl$_2$, 1 mM DTT, 100 μg/ml bovine serum albumin, 0.1 mM each of dGTP, dCTP, dATP, 0.1 mM ^{3}H-dTTP (198 cpm/pM), 1 μl of HeLa cell extract (approximately 0.1 units of DNA polymerase) or 0.03 units of purified enzyme, 200 μg/ml of activated calf thymus DNA and exogenous nucleic acid as specified. Activated calf thymus DNA was prepared by exposing the DNA (2 mg/ml) to 40 ng/ml of pancreatic DNase (Worthington Biochemical Corp., Freehold, NJ) in a solution containing 50 mM Tris pH 7.5, 7.5 mM MgCl$_2$ and 0.5 mg BSA. After 15 minutes at 37°C, EDTA was added to 40 mM, the solution was heated for 5 minutes at 77°C and immediately cooled in an ice bath. The polymerase reaction mixture was incubated at 35°C for 60 minutes. Acid insoluble radioactivity was determined by precipitation of the mixture with cold 5% TCA onto Whatman GF/C filter papers which were washed with 5% TCA, 1 N HCl, 95% ethanol, dried and counted in scintillation fluid. One unit of DNA polymerase was defined as the amount of DNA polymerase that would incorporate 1 nmol of ^{3}H-dTMP into acid insoluble product in 1 hour.

Results

The Effect of Vesicular Stomatitis Virus Coinfection into Adenovirus
Infected Cells in Tissue Culture. The specific aim of this disserta-
tion is to discover whether VSV can specifically inhibit eukaryotic DNA
synthesis using the Ad2 in vitro DNA replication assay as a model system.
To establish the relevance of an in vitro model, it must be determined
if VSV is able to affect the course of Ad2 infection in vivo. To ex-
amine this, VSV was coinfected into Ad2 infected HeLa cells in tissue
culture with the assumption that if VSV is able to dominate in coin-
fected cells, it may affect Ad2 DNA replication in vitro.

Nearly confluent HeLa cells were infected with Ad2 at an MOI
of 10, and incubated for 8 hours at 37°C. After incubation, cells
were coinfected with VSV (serotype Indiana) at a MOI of 10 and in-
cubated for an additional 4 hours in the presence of ^{35}S-methionine.
Cells were then pelleted, washed and lysed. Cell lysates were electro-
phoresed into a 12% SDS-polyacrylamide gel and autoradiographed. From
the representative autoradiograph in Figure 5, VSV proteins predominate
in extracts prepared from coinfected cells to the exclusion of Ad2 and
host cellular proteins. Cells which were infected with Ad2 but not VSV
show Ad2 specific protein synthesis at 18 hours p.i. Similar experi-
ments in which VSV was coinfected into cells at various times during
the early phase of Ad2 infection (1 hour to 12 hours p.i.) produced
identical results. These studies show that VSV can arrest Ad2 infec-
tion and shut down host cellular protein synthesis, whereas the
presence of early phase Ad2 products within these cells had no im-
mediately discernable effect on VSV expression.

Figure 5. <u>Protein Synthesis in Ad2-VSV Coinfected HeLa Cells</u>. Ex-
tracts of HeLa cells infected with VSV (A), Ad2 plus VSV (B) Ad2 (C)
and uninfected cells (D) which were incubated in media containing
^{35}S-methionine for 4 hours. Lysates of each cell population were made,
subject to SDS-PAGE and autoradiographed. The positions of the bands
corresponding to the VS viral proteins L, N, NS, G and M are indicated.
In cells coinfected with Ad2 and VSV, VSV proteins predominate to the
exclusion of host cellular and Ad2 proteins.

VSV SHUT-OFF OF ADENOVIRUS
PROTEIN SYNTHESIS
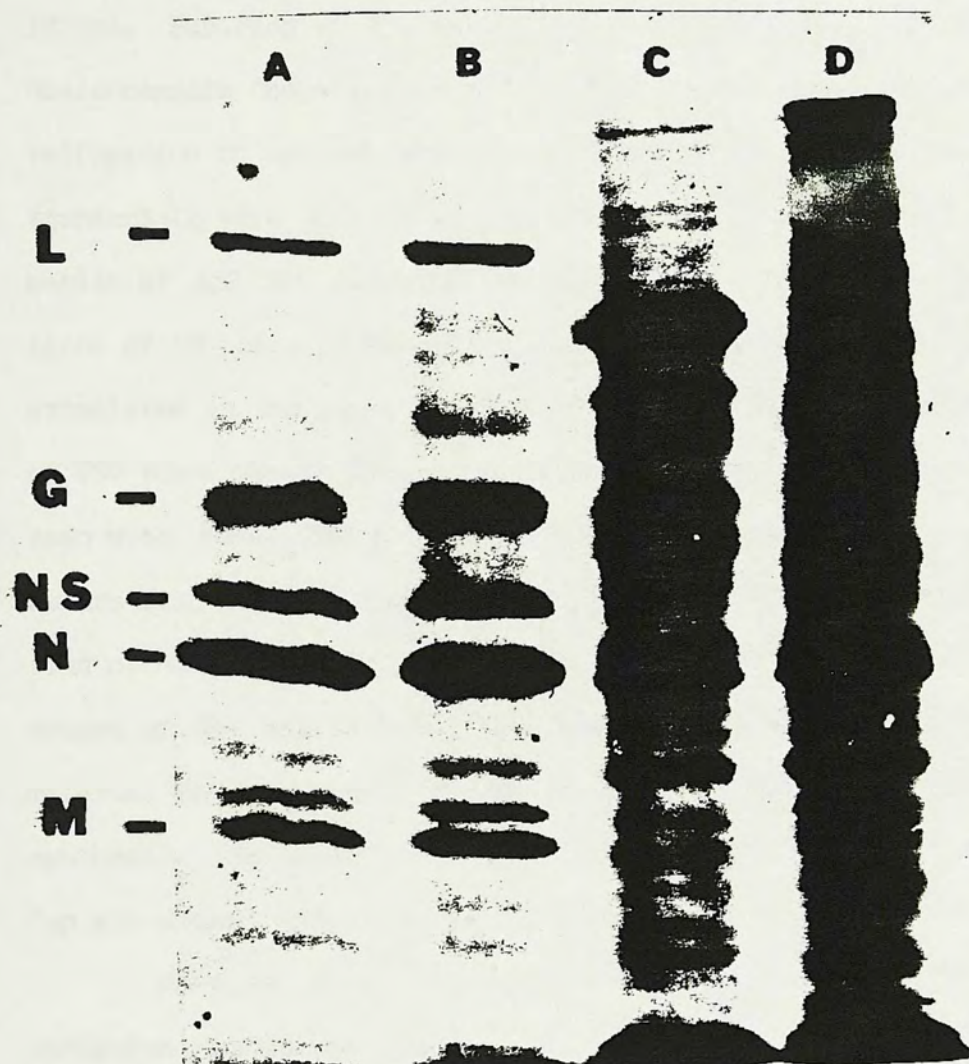
The Effect of Exogenously Added Unfractionated VSV RNA and Bovine tRNA
on DNA Synthesis. McGowan and Wagner (1981) have shown that upon in-
fection, VSV must undergo primary transcription to inhibit cellular DNA
synthesis. Therefore, plus strand VSV RNA transcribed in vitro was
tested for its ability to inhibit the replication of Ad2 DNA in nuclear
extracts prepared from Ad2-infected HeLa cells (Challberg and Kelly,
1979b). Purified wt VSV was in vitro transcribed at 31°C for 4 hours.
Nucleocapsids (negative strand template) were removed by velocity cen-
trifugation to prevent possible formation of double stranded RNA. These
transcripts were added into the Ad2 DNA replication assay, and the ki-
netics of Ad2 DNA synthesis in the presence of increasing concentra-
tions of VSV plus strand RNA are shown in Figure 6. DNA synthesis was
stimulated in the presence of relatively low concentrations (12 ng/μl)
of VSV plus strand RNA. A stimulation of Ad2 DNA synthesis was also
seen with other RNA tested in this study and has been reported by
others (van der Vliet et al., 1984; see Figure 7). As the concentra-
tion of VSV transcripts was increased in the reaction mixture, a de-
crease of the relative incorporation of ^{32}P-dCMP into acid insoluble
material was observed. At 240 ng/μl, there was 95% inhibition of DNA
synthesis. The kinetics of viral nucleic acid synthesis were similar
for all concentrations tested despite the degree of inhibition observed.

Previous studies have implicated leader RNA as the VSV tran-
scription product responsible for the inhibition of cellular nucleic
acid synthesis (Grinnell and Wagner, 1984; McGowan et al., 1982; Weck
et al., 1979). Therefore, another exogenously added eukaryotic small
RNA, bovine tRNA, was tested for its ability to inhibit Ad2 DNA syn-
thesis (Figure 6). Bovine tRNA is larger in size than leader RNA and

Figure 6. <u>The Kinetics of Ad2 DNA Synthesis in the Presence of Exo-genous (A) In Vitro Transcribed VSV Plus Stranded RNA or (B) Bovine tRNA.</u> Reaction mixtures of 50 µl each were incubated with (▼) 12 ng/µl, (▽) 36 ng/µl, (◊) 120 ng/µl, (■) 160 ng/µl, (□) 200 ng/µl, (o) 240 ng/µl and in the absence (♦) of added RNA. Equal aliquots were removed at the indicated time points, TCA precipitated and washed. The amount of ^{32}P-CMP incorporated into each precipitate was determined by Cerenkov counting.
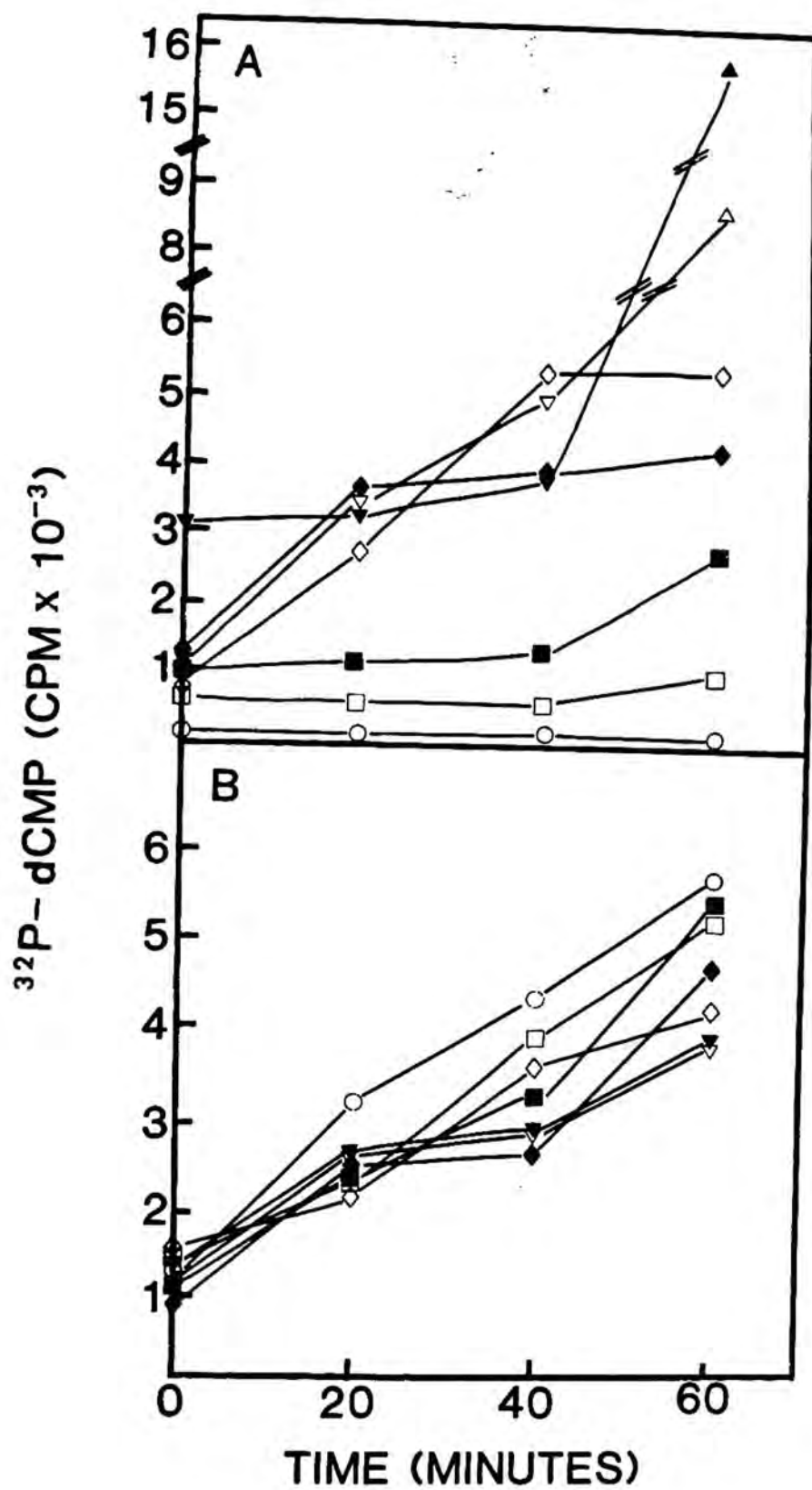
has a complex secondary structure. Addition of bovine tRNA did not vary the reaction by more than 20% over the range of concentrations tested. These results indicate that a specific transcription product of VSV inhibits Ad2 DNA synthesis in vitro. This study was not designed to measure the specific initiation of Ad2 DNA replication, but total Ad2 nucleic acid synthesis. A more sophisticated design was used in the following experiments to determine that a single transcription product of VSV will inhibit the specific replication of Ad2 DNA in vitro.

Comparison of the Ability of VSV Wt Leader, DI Leader, VSV mRNA and Yeast RNA to Inhibit Ad2 DNA Replication. Purified wt leader RNA, the small RNA made by defective interfering particle DI-011 (DI leader RNA), oligo(dT) selected VSV mRNA, and torula yeast RNA were individually tested for their ability to block the specific replication of adenovirus DNA. Ad2 template was cleaved with the restriction endonuclease HindIII prior to its addition into the assay (Challberg and Kelly, 1979a). Reactions were incubated for 60 minutes, and the products resolved as discussed (see Material and Methods). As shown in Figure 7, HindIII digestion of Ad2 DNA yields thirteen fragments. Two contain the terminal sequences and are relatively small in size [2.7 and 1.0 kilobase pairs (Kbp)] in comparison to the internal fragments. These two fragments contained 95% of incorporated isotope, but together only represent 12% of the total genome by weight. Preferential incoroation of isotope into these fragments is indicative of the specificity of DNA synthesis and serves as reasonable evidence to the assumption that the in vitro system mimics accurate adenovirus DNA replication.

Figure 7. The Effects of Purified VSV Transcripts and Yeast RNA on Ad2
In Vitro DNA Replication. Ad2 template was replicated in the presence
of the following RNA added at 1.5 ng/μl into a 20 μl reaction mixture
and the products analyzed on a 0.8% agarose gel: C = no exogenous
nucleic acid, L = plus stranded VSV leader RNA, D = the small RNA made
by defective interfering particle, DI-011 (DI leader RNA), M = oligo(dT)
selected plus stranded VSV mRNA, Y = Torula yeast RNA. P = replication
of Ad2 template which had been previously digested by proteinase K with
no exogenous nucleic acid added.

THE EFFECT OF SMALL RNA'S ON Ad2 REPLICATION

C L D M Y P

2.7Kbp ➤

1.0 Kbp ➤

Ad2 HIND III

2.7 ——————————————— 1.0
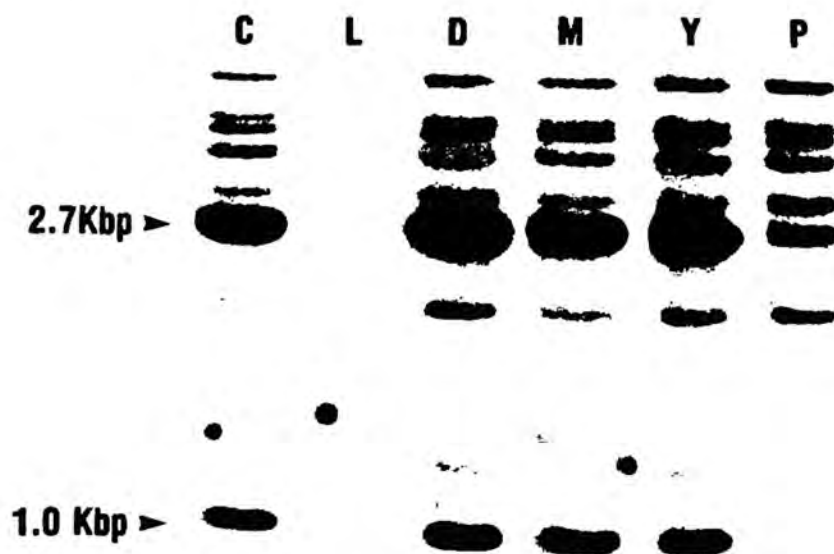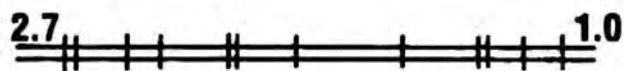
Table III.   The Effect of Purified RNA on Ad2 DNA Replication.

RNA Added	Concentration ng/μl	nM	Percent of Control Replication
Wild-type Leader	1.5	96	5
	5.0	318	1
	15.0	955	1
DI Leader	1.5	97	106
	5.0	325	96
	15.0	974	99
VSV Poly(A) RNA	1.5	–	89
	5.0	–	106
	15.0	–	104
Yeast RNA	1.5	–	100
	5.0	–	127
	15.0	–	124

Shown is a comparison of the ability of various RNA to inhibit Ad2 in vitro replication. The amount of DNA synthesis which occurred was quantitated as described in Materials and Methods. The amount of Ad2 specific DNA synthesis that occurred is given as the percent of replication which occurred in the absence of exogenous RNA.

Specific replication was not observed when proteinase K treated Ad2 template was assayed attesting to the requirement for the terminal protein. Next, purified RNAs were tested for their ability to inhibit the specific incorporation of ^{32}P-dCMP into the terminal fragments of adenovirus template. Wt leader RNA completely inhibited the specific replication of the terminal fragments at a concentration of 1.5 ng/µl (97 nM). Quantitation of the data obtained in these and similar experiments is presented in Table III. DI leader RNA, oligo(dT) selected VSV mRNA, and torula yeast RNA did not inhibit DNA synthesis at concentrations ten times higher than wt leader RNA.

These data directly imply that wt leader RNA is the VSV transcript responsible for inhibition of Ad2 DNA synthesis in vitro. Inhibition is a sequence-specific phenomenon as demonstrated by the inability of DI-011 leader RNA to produce inhibition, even though it is 60% homologous in sequence to wt leader and was purified in an identical manner.

Inhibition of Ad2 DNA Replication In Vitro Using Synthetic Oligodeoxy-nucleotides. Due to the difficulties, expense and amount of time required to produce large quantities of leader RNA, synthetic oligo-deoxynucleotides analogous to the sequence of VSV leader RNA and DI-011 leader RNA were obtained. Their sequences are as follows:

J7 5' -   ACGAA GACAAACAAA CCATTATTAT CATTAAAAGG CTCAGGAGAA AA - 3'

J8 5' - GATCCACGAA GACAAACAAA CCATTATTAT CATTAAAAGG CTCAGGAGAA AAG - 3'

J10 5' - GATCCACGAA GACCACAAAA CCAGATAAAA AATAAAAACC ACAAGAGGGT CG - 3'

J8 and J10 possess five extra n.t. at their 5' ends and one extra at

their 3' ends which are not found in the sequence of VSV leader RNA and DI leader RNA, respectively. These extra nucleotides were useful for cloning of these synthetics into plasmid vectors for future experiments.

As shown in Figure 8, J7 and J8 are equally as effective as VSV leader RNA in producing inhibition of Ad2 in vitro DNA replication. In contrast, J10 did not produce significant inhibition of Ad2 DNA synthesis. In these experiments, the presence of extra n.t. at the 5' and 3' ends of J8 and J10 did not interfere with their inhibitory properties.

A series of experiments using these synthetic molecules were performed to establish the efficacy of the inhibition they produced and to better understand the process of inhibition. The first was the titration of the inhibitory effect of J7 on Ad2 DNA replication (Figure 9). It was determined that 1.5 ng/µl of J7 inhibits the specific incorporation of isotope into the terminal HindIII fragments of the Ad2 genome. This concentration of J7 (or J8) is used in all subsequent experiments as the minimum concentration required for inhibition. At 2 ng/µl and above, which is still not enough nucleic acid to produce detectable inhibition with DI leader RNA or J10, J7 also inhibits nonspecific DNA synthesis of the internal Ad2 HindIII fragments.

From these results, it is not clear if the inhibition of specific Ad2 DNA replication and random DNA synthesis occur by separate mechanisms or a common process. It would be helpful to determine the kinetics of inhibition in order to establish if inhibition is a competitive, a noncompetitive or an uncompetitive phenomenon. Kinetic studies unfortunately are not possible with purified proteins or crude extracts because the proteins required for Ad2 DNA replication

Figure 8. The Effect of Synthetic Oligodeoxynucleotides on Ad2 DNA Replication In Vitro. Ad2 HindIII cleaved template was replicated in the presence of: (A) no exogenous nucleic acid, (B) J7, (C) J8, (D) J10. Synthetics were used at a concentration of 1.5 μg/ml. The products were analyzed on a 0.8% agarose gel as described in Materials and Methods.

THE EFFECT OF SYNTHETIC OLIGODEOXYNUCLEOTIDES ON Ad2 REPLICATION
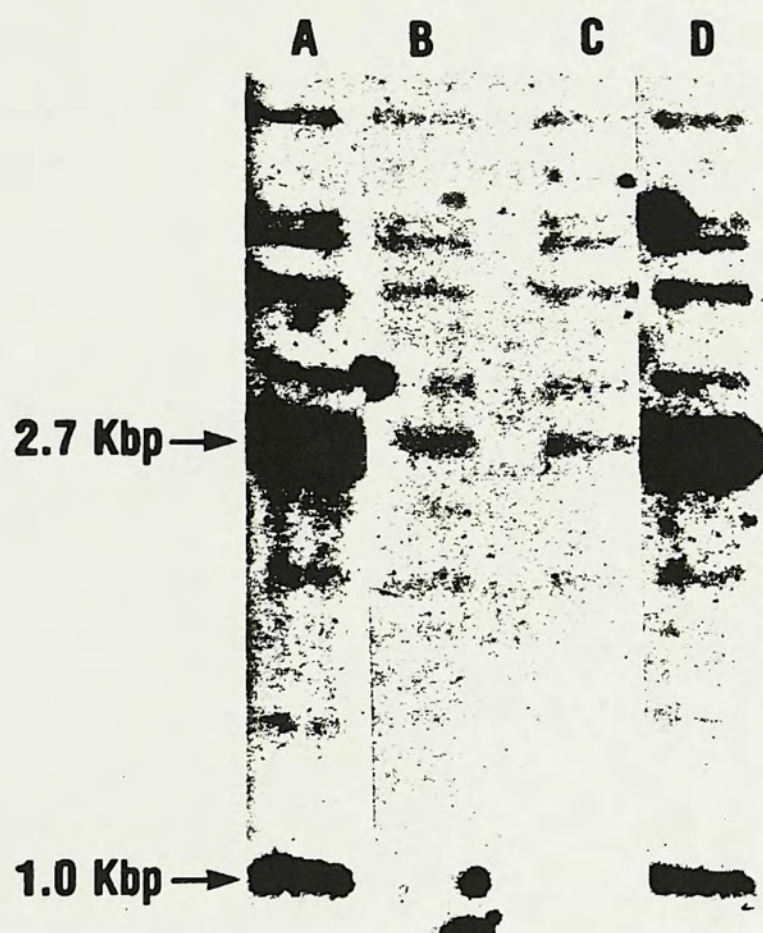
Figure 9. <u>Titration of J7 in Ad2 DNA Replication Reactions In Vitro</u>. 0, 10, 20, 30, 40 and 50 ng of J7 were added into Ad2 <u>in vitro</u> DNA replication reactions as shown. At 30 ng (1.5 ng/μl reaction mixture) specific Ad2 DNA replication is completely inhibited. At 40 ng (2 ng/μl reaction mixture) and above nonspecific Ad2 DNA synthesis is also greatly reduced.

**TITRATION OF J7 INTO Ad2
REPLICATION REACTIONS**

0   10   20   30   40   50

2.7Kbp ►

1.0Kbp ►
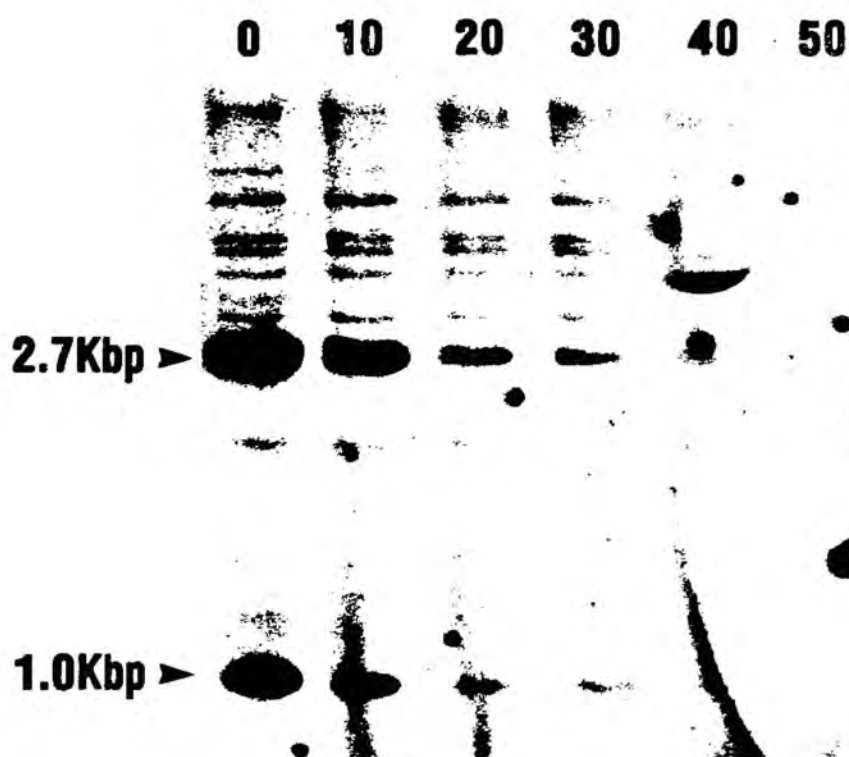
are not present in saturating amounts nor can their concentrations be varied to any significant degree.

Inhibition of Ad2 DNA Replication by Synthetic Nucleotides Representing Various Regions of VSV Leader RNA. Synthetic oligodeoxynucleotides analogous to portions of VSV leader RNA were tested for their inhibition of Ad2 DNA replication in vitro. The sequences of these synthetics in relation to VSV leader RNA are shown in Table IV.

The synthetic oligodeoxynucleotide J5 which contains the A-rich sequence analogous to nucleotide positions 8 to 19 of VSV leader RNA produced the largest degree of inhibition. Part of this sequence (n.t. 18 to 24) was shown by Grinnell and Wagner (1984) to be important for inhibition of RNA polymerase II and III activities in vitro indicating that this region is likely the active site of VSV leader RNA for the inhibition of host cell nucleic acid synthesis. This data also suggests that the mechanism of inhibition of DNA and RNA synthesis may act through a similar pathway.

J1 and J2 which represent the central and the 3' terminal sequences of VSV leader RNA, respectively did not significantly inhibit Ad2 DNA replication in this assay. The inability of J1 to inhibit Ad2 DNA replication in comparison to the results with J5 suggests that the region of leader RNA between n.t. 8 to 16 is important for shut-off of Ad2 specific DNA synthesis.

Hybridization of VSV Leader RNA to the Ad2 Template. One possible explanation for the ability of VSV leader RNA to inhibit Ad2 DNA synthesis is that leader RNA may be able to hybridize to the origin of replication of Ad2 DNA, thereby blocking the protein-nucleic acid interactions

Table IV.

Shown is a comparison of the ability of various synthetic oligodeoxy-
nucleotides to inhibit Ad2 DNA replication. The amount of Ad2 specific
DNA synthesis which occurred is given as the percent of replication
that occurred in the absence of exogenous nucleic acid. The position
of each synthetic oligodeoxynucleotide in relation to VSV leader RNA is
shown. All sequences are presented in their 5' to 3' orientation.
Replication reactions were performed as described in Materials and
Methods for Ad2 DNA replication using purified proteins. Forty µg
(2 µl) of Ad2 infected HeLa cell nuclear extract were substituted for
the purified protein fractions.

Table IV. Inhibition of Ad2 DNA Replication by Small Synthetic Oligo-
nucleotides Analogous to Short Regions of VSV Leader RNA.

Synthetic Oligodeoxynucleotide	Concentration ng/μl	μM	Percent of Replication
J1	5	0.88	100
	10	1.75	104
	15	2.62	105
	25	4.37	69
J2	5	0.93	78
	10	1.85	77
	15	2.78	79
	25	4.63	78
J5	5	0.68	35
	10	1.35	33
	15	2.03	31
	25	3.38	16

```
                              10        20        30        40
VSV leader RNA   5'-ACGAAGACAAACAAACCAUUAUUAUCAUUAAAAGGCUCAGGAGAAAA-3'

J1                              CATTATTATCATTAAAA

J2                                               AAAGGCTCAGGAGAAA

J5                   CAAACAAACCATTATTATCATT
```

which occur at the viral origins during replication. A comparison of the sequence of leader RNA with the terminal sequence of Ad2 DNA produced no homologous regions of greater than 5 n.t. in length.

Ad2 template (terminal protein - DNA complex) was purified as described in Materials and Methods and blotted onto nitrocellulose paper. ^{32}P-labeled J8 was probed to these blots which were subsequently washed and autoradiographed (Figure 10). No significant hybridization was observed. Also, no hybridization was observed when alkali denatured Ad2 template was probed with J8. Due to the greater stability of DNA-DNA double-strands compared with RNA-DNA double-strands, this experiment was not repeated with leader RNA. Proteins present in the HeLa cell extract may, however, permit such an interaction to occur in vitro. Therefore, the possibility of leader RNA binding to the Ad2 template cannot be ruled out at this time.

Stability of Wt Leader RNA in the Ad2 DNA Replication Reaction. If wt leader RNA is complexed with proteins or nucleic acids needed to replicate adenovirus in vitro, then leader could not be degraded by nucleases within the extract. Therefore, the stability of wt and DI leader RNA in the replication system were assayed. Two ng/μl of purified radiolabeled wt or DI leader RNA were added into adenovirus DNA replication reactions in the absence of ^{32}P-dCTP (Figure 11). After 60 minutes of incubation, 64% of wt leader RNA but only 22% of DI leader RNA remained intact. This result implies that wt leader RNA interacts with a component of the replication extract which protects it from RNase attack. The synthetic oligodeoxynucleotides, J8 and J10, were found to be greater than 90% stable after 60 minutes in nuclear extracts under identical conditions, indicating that the selective stability of wt

Figure 10. <u>Hybridization of J8 to the Ad2 Template</u>. Increasing amounts of native Ad2 protein-DNA template and heat denatured template bound to nitrocellulose paper was probed with 5' end ^{32}P-radiolabeled J8. Upon autoradiography, it can be seen that J8 does not significantly hybridize to Ad2 template DNA. Twenty-five ng of a synthetic oligodeoxynucleotide complementary to J8 was used as a positive control and 25 ng of calf thymus DNA as a negative control.

HYBRIDIZATION OF J8 WITH Ad2 TEMPLATE DNA

NATIVE TEMPLATE

DENATURED TEMPLATE

← 25 ng →
← 50 ng →
← 100 ng →
← 200 ng →

←(+) CONTROL →
(−) CONTROL
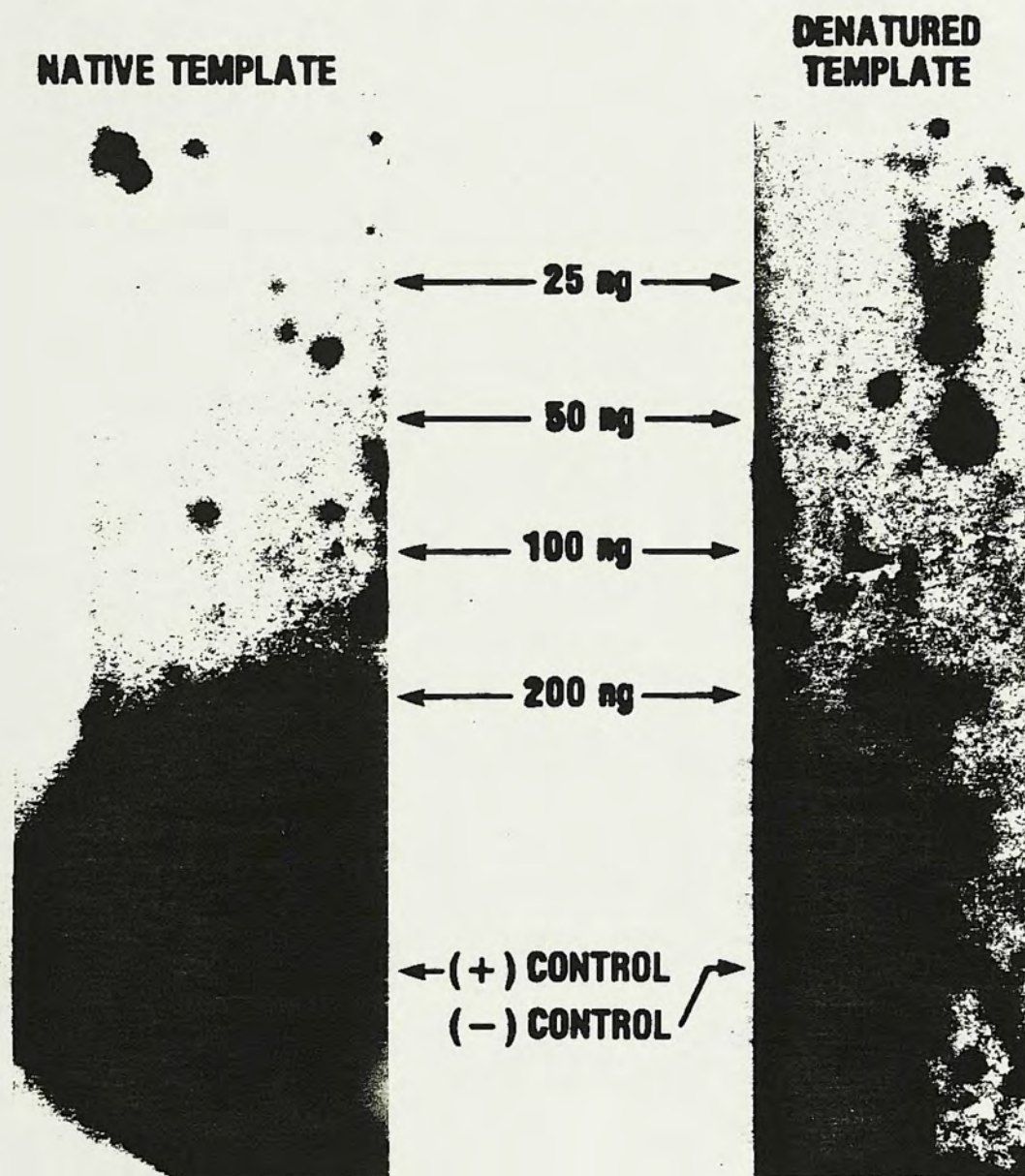
Figure 11. <u>The Stability of Wild-type and Defective Interfering Parti-</u><u>cle Leader RNA in the Replication Extract.</u> Ten ng of ^{32}P-UMP labeled wt or DI leader RNA were incubated under typical reaction conditions with the replication extract. Equal aliquots were removed at 15 minute intervals and electrophoresed into a 20% acrylamide, 8 M urea gel. Sixty-four percent of full-length wt leader RNA and 22% of full-length DI leader RNA remained intact after 60 minutes of incubation as determined by densitometric scanning of the autoradiogram.

THE STABILITY OF LEADER RNA
IN THE REPLICATION REACTION

Wt LEADER RNA

0 '   15 '   30 '   45 '   60 '

DI-011 LEADER RNA

0 '   15 '   30 '   45 '   60 '

leader RNA is not the sole reason for the inhibition of Ad2 *in vitro* DNA replication.

This component may be the target of leader RNA in the replication assay, and its interaction with leader RNA result in the observed inhibition of specific Ad2 DNA synthesis. Experiments were undertaken to identify this component in the hope of determining the mechanism of inhibition.

The Association of J7 with a Factor Present in the Nuclear Extract.

From VSV leader RNA's surprising stability in the nuclear extract, it seemed likely that it was complexed with a factor that may be required for Ad2 DNA replication *in vitro*. To demonstrate a possible association, 1 ng of radiolabeled J7 was incubated with 100 ng of nuclear extract for 15 minutes at 31°C, layered onto a 5% to 25% sucrose gradient and centrifuged at 285,000 xg for 12 hours. The gradient was collected, and the fractions Cerenkov counted. A graphic representation of the results (Figure 12) shows that in gradients which contained J7 plus extract, J7 migrated further down the tube when compared to gradients which contained only J7. Again, these results suggest that J7 is associated with a factor present in the nuclear extract. Fractions from the gradient which contained the J7 factor complex were pooled, dialyzed against replication buffer to remove sucrose and added into replication reactions in an attempt to overcome the inhibition produced by leader RNA. No reversal of inhibition, however, was observed (Table V). When this experiment was repeated with VSV leader RNA, RNases inherent to the extract digested the RNA and distributed radiolabel throughout the gradient.

To identify the factor from nuclear extracts which complexes

Figure 12.   Association of J7 with a Component of the Nuclear Extract.
One ng of 5' end ^{32}P-radiolabeled J7 and 1 ng radiolabeled J7 which had
been incubated in 100 ng of Ad2 infected HeLa cell nuclear extract for
15 minutes were centrifuged into a 5-25% sucrose gradient. Upon frac-
tionation of the gradient and Cerenkov counting of each fraction, it
can be seen that J7 in the presence of nuclear extract migrated further
into the gradient than J7 alone suggesting that J7 was closely as-
sociated with a component of the nuclear extract.

Sucrose Gradients of J7—Extract
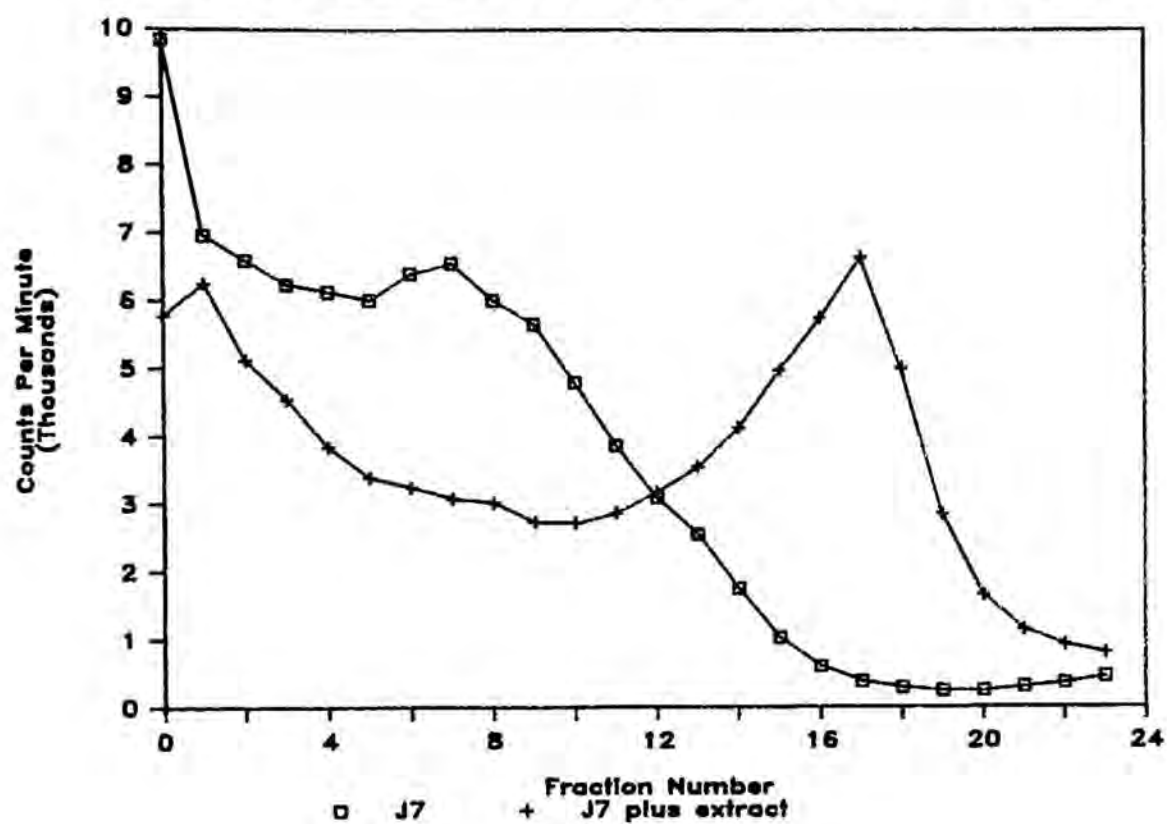
Table V. Reconstitution Attempts of Ad2 DNA Replication Inhibited by Leader RNA.

Fraction Added	Volume Added	Final Concentration	Percent of Replication
none	0	--	100
<u>Sucrose gradient fractions</u>	1 μl	--	2
	5 μl	--	4
	10 μl	--	2
<u>Fraction eluted from J7-linked sepharose column eluted at:</u>			
50 mM	1 μl	--	2
	10 μl	--	1
200 mM	1 μl	--	7
	10 μl	--	2
500 mM	1 μl	--	6
	10 μl	--	2
1 M	1 μl	--	1
	10 μl	--	1
<u>Nuclear factor I (200 μg/ml)</u>	1 μl	0.21 μM	5
	5 μl	1.05 μM	7
	10 μl	2.10 μM	2
	12 μl	2.52 μM	9

Ad2 replication assays were performed in the presence of the minimal amount of VSV leader RNA required to produce maximal inhibition of Ad2 replication. The amount of Ad2 replication which occurred is given as the percent of replication that occurred in the absence of leader RNA and exogenous substrate.

with J7, a J7 linked agarose column was constructed. ^{32}P-labeled J7 was coupled via carbodiimide catalysis to AH sepharose 4B with a six carbon spacer group according to the procedure of Pharmacia (1983). After extensive washes and equilibration with replication buffer, the efficiency of coupling was determined to be 17%. One-hundred μl of Ad2 infected HeLa cell nuclear extract was added to a column of 100 μl of J7-coupled sepharose (0.5 μg) which was incubated at room temperature for 30 minutes. The column was washed with replication buffer and eluted with increasing concentrations (0.05 M to 1.0 M) of phosphate buffer. Fractions were collected, dialyzed against replication buffer and added into inhibited replication reactions. No reconstitution of Ad2 specific DNA synthesis was observed (Table V).

A similar column was constructed without coupled nucleic acid. Nuclear extract was added, fractions were collected and treated as before. Aliquots of each fraction were electrophoresed next to aliquots from fractions collected off the J7 containing columns into 12% SDS-polyacrylamide gels. Upon silver staining, no specific bands were present in the fractions from the J7 coupled column that were not in corresponding fractions from the DNA deficient column (Figure 13). From the numerous bands of protein in each lane, however, it is difficult to discern any single species. Extensive washes of the sepharose did not significantly reduce the binding of these extraneous proteins. Additional experiments using the J7-coupled sepharose were performed to demonstrate that J7 bound to a protein(s) that affects Ad2 DNA replication.

Five μl of J7-coupled sepharose were incubated in nuclear extract for 15 minutes then removed by low speed centrifugation. Ad2 in

Figure 13.   <u>Proteins Present in Fractions Eluted from Agarose Columns</u>
<u>Incubated with Ad2 Infected HeLa Cell Nuclear Extract.</u>   Ad2 infected
HeLa cell nuclear extract was incubated with either J7 linked agarose
(B, D, F) or agarose alone (C, E, G) and eluted with increasing con-
centration of sodium phosphate buffer.   Eluted fractions were electro-
phoresed into a 12% SDS-polyacrylamide gels which was subsequently
silver stained.   The contents of each lane are as follows: molecular
weight standards (A), 50 mM sodium phosphate eluted fractions (B and
C); 200 mM sodium phosphate eluted fractions (D and E); 500 mM sodium
phosphate eluted fractions (F and G).

FRACTIONS ELUTED FROM SEPHAROSE COLUMNS
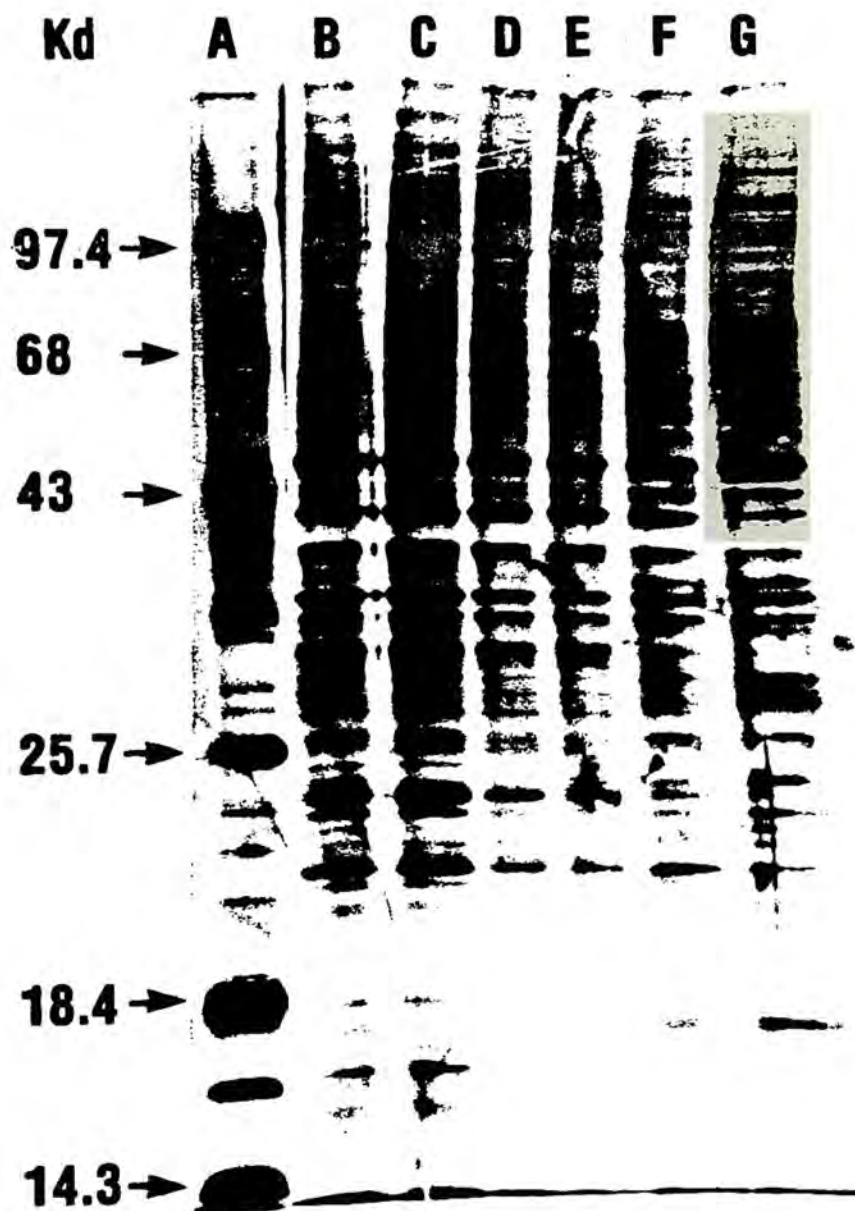
Table VI. The Effect of AH-sepharose-4B Treatment of Nuclear Extract on Ad2 In Vitro DNA Replication.

Sepharose Tested	J7 ng/µl	nM	Percent of Ad2 DNA Replication
None	--	--	100
J7- linked	1.0	64	5
Unlinked	--	--	6
J7-linked BSA + tRNA treated	1.0	64	4
Unlinked BSA + tRNA treated	--	--	6

J7-linked or unlinked AH-sepharose-4B was incubated with Ad2 infected HeLa cell nuclear extract then removed by centrifugation in the hope that J7 would bind to and remove a factor required for Ad2 DNA replication. The amount of linked J7 used was determined as described in Materials and Methods. The percent of Ad2 DNA replication that occurred is given as the amount of DNA synthesis which occurred in the absence of sepharose pretreatment.

vitro DNA replication reactions were performed using this extract and no specific Ad2 DNA synthesis occurred suggesting that J7 bound and removed a factor required for Ad2 DNA replication (Table VI). Unfortunately though, no Ad2 DNA synthesis occurred using 5 µl of extract which had been preincubated with uncoupled sepharose indicating that the sepharose nonspecifically removed protein required for replication or interfered in some other way with the replication process. To prevent nonspecific binding, both J7-coupled and uncoupled sepharose was incubated with bovine serum albumin and tRNA. The sepharose was washed extensively, and the above experiments repeated. Again, nuclear extracts pretreated with uncoupled sepharose were unable to produce Ad2 specific DNA synthesis (Table VI). From these results, it was decided to pursue the identity of the factor which associates with leader RNA (or analogous synthetic oligonucleotides) through alternative methods.

Reconstitution of Ad2 DNA Replication in the Presence of VSV Leader RNA with Extracts Prepared from HeLa Cells. Previous reports (Grinnell and Wagner, 1985; McGowan and Wagner, 1981; Weck et al., 1979) have measured the ability of VSV leader RNA to inhibit nucleic acid synthesis. Reconstitution, however, is essential to identify the factor targeted by leader RNA which is responsible for inhibition, and to prove that the inhibition observed is not due to a toxic effect.

Reconstitution of VSV leader RNA inhibited replication was tested for by the addition of supplemental Ad2-infected nuclear extract, uninfected nuclear extract and Ad2 infected cytoplasmic extract, a by-product of the preparation of the nuclear extract which is enriched for Ad2 early gene products (Philip J. Rosenfeld, personal communication). A representative autoradiogram of these results is shown in

Figure 14. Reconstitution of VSV Leader RNA Inhibited Ad2 DNA Replica-tion with HeLa Cell Extract. HindIII cleaved Ad2 template was in vitro replicated as previously described with the following additions: (A) no additions, (B-H) 1.5 ng/µl of leader RNA, (C) 20 µg and (D) 40 µg of Ad2 infected HeLa cell cytoplasmic extract, (E) 10 µg and (F) 20 µg of Ad2 infected HeLa cell nuclear extract, (G) 20 µg and (H) 40 µg of uninfected HeLa cell nuclear extract. Reaction products were analyzed on a 0.8% agarose gel and autoradiographed.

RECONSTITUTION OF INHIBITED REPLICATION REACTIONS WITH HELA CELL EXTRACT
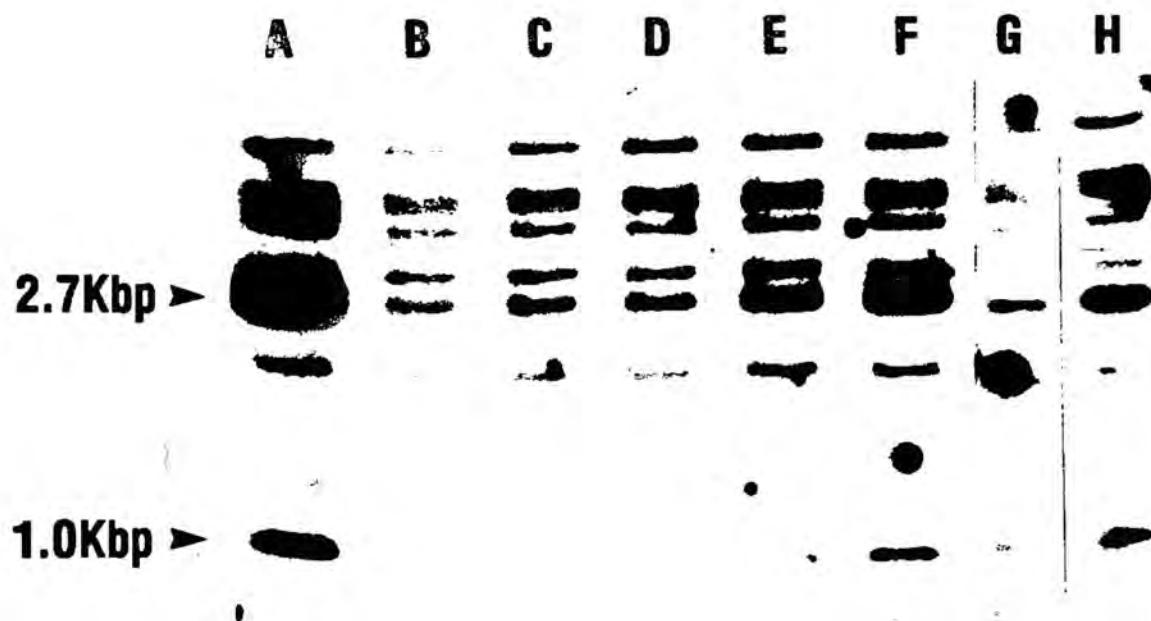
Figure 14. The addition of excess Ad2 infected or uninfected nuclear extract overcame the inhibitory effects of leader RNA and permitted specific replication to occur, albeit to a lesser degree than in control reactions. Cytoplasmic extract, in contrast, did not restore specific replication.

These findings could be interpreted to suggest that a host cell protein common to both the infected and uninfected extracts is capable of overcoming the inhibition produced by leader, or that inhibition was reversed by a nonspecific mechanism common to each. Assuming that the mechanism of reconstitution is a specific process, the only cellular protein required for replication of the terminal Ad2 HindIII fragments is NFI. Reconstitution was not possible with up to a 25-fold excess of purified NFI over inhibitor concentration (Table V). The preparation of NFI was determined to be active by its ability to stimulate pTP-dCMP complex formation in the presence of Ad2 infected HeLa cell cytoplasmic extract as described by Lichy et al. (1981). If the target of leader RNA was NFI or an as yet unidentified factor which copurifies with NFI, then a 25-fold excess should have been able to restore some degree of replication. Also, leader RNA does not contain the consensus sequence required for NFI binding (see Figure 19). Consequently, it appeared that the target of VSV leader RNA and the factor responsible for reconstitution of inhibited replication reactions in nuclear extracts is not NFI.

Noncovalent Association of the Synthetic Oligodeoxynucleotide J8 with a Protein(s) of HeLa cell Extracts. Experiments were performed to identify a factor in nuclear extracts which may bind to the sequence of leader RNA. J8 was 5' end-labeled with ^{32}P and incubated with 3 μl (60

Figure 15. Noncovalent Association of J8 with Proteins of the HeLa
Cell Extract. The following substrates were added to replication buf-
fer, incubated for 60 minutes at 31°C, subjected to 12% PAGE in the
absence of SDS (nondenaturing conditions) and autoradiographed: (A)
10 μCi of γ32P-ATP, (B-C) 10 ng of 5' end ^{32}P-radiolabeled J8,
(A, C, H, I) 60 μg of Ad2 infected HeLa cell nuclear extract, (D)
60 μg of uninfected HeLa cell nuclear extract, (E) 30 μg of Ad2
infected HeLa cell cytoplasmic extract, (F) 10 μg of a purified
protein fraction containing the Ad2 DNA polymerase and the precursor of
the Ad2 terminal protein, (G) 10 μg of the Ad2 DNA binding protein
and 50 ng of nuclear factor I, (H) 10 ng of a 5' end ^{32}P-radiolabeled
synthetic oligodeoxynucleotide analogous to the first 46 n.t. of mouse
hepatitis virus strain A59 leader RNA which is unrelated to the se-
quence of VSV leader RNA, (I) 10 ng of 5' end ^{32}P-radiolabeled J10.
The interface of the stacking gel and running gel retained some radio-
activity and is indicated by an arrow (top). Roman numerals indicate
the positions of the slower migrating complex (I), faster migrating
complex (II), free radiolabeled J8 (III) and free radiolabeled nucleo-
otides (IV).

ASSOCIATION OF J8 WITH PURIFIED PROTEINS AND HELA CELL EXTRACTS ON A NONDENATURING GEL
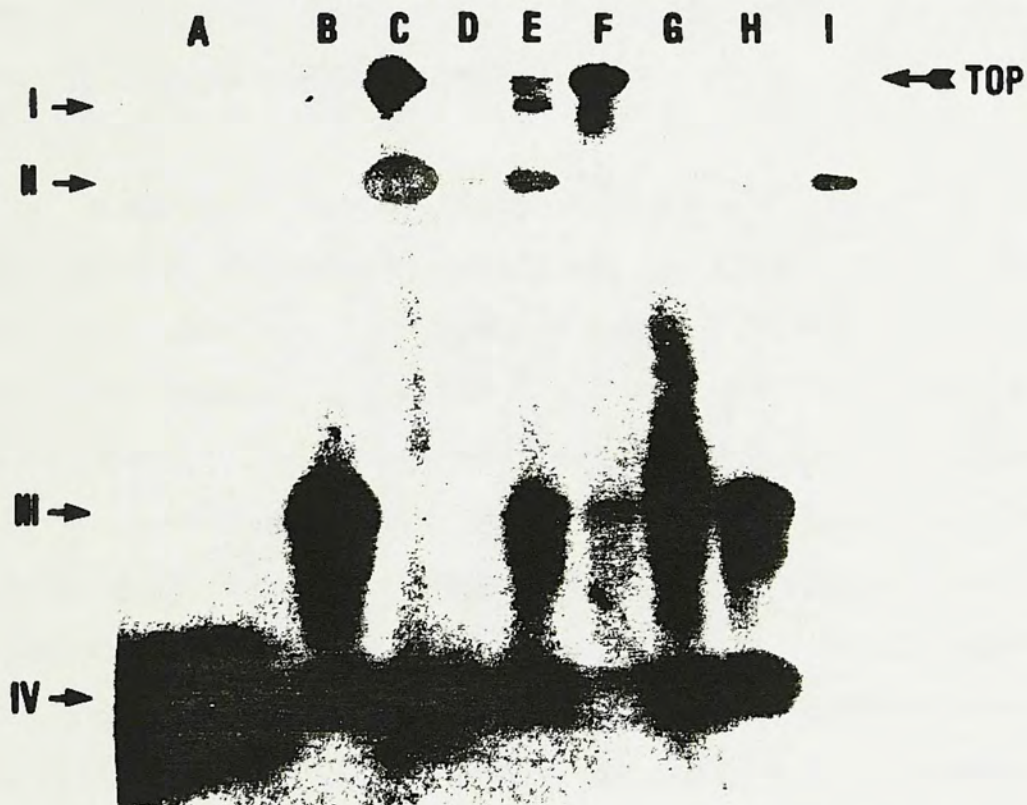
μg) of extract under replication conditions for 60 minutes. Bromophenol blue and glycerol were added to the mixture which was electrophoresed into a nondenaturing 12% polyacrylamide gel. Dye was electrophoresed to the bottom of the gel and upon autoradiography (Figure 15), J8 in the presence of either Ad2 infected or uninfected HeLa cell nuclear extract migrated at a slower mobility (Figure 15, band II) than free J8 (Figure 15, band III). J10 in the presence of Ad2 infected HeLa cell nuclear extract also migrated to a similar position (Figure 15, band II).

A synthetic oligonucleotide unrelated to the sequence of leader RNA, incubated with nuclear extract did not migrate at a different mobility on this gel. Interestingly, in the presence of 30 μg of cytoplasmic extract, J8 migrated to two separate positions (Figure 15, bands I and II). One is similar to the position of J8 after incubation with nuclear extract and is presumably due to the presence of nuclear protein in the cytoplasmic extract. The second band is also observed in nuclear extract which had been incubated with γ32P-ATP suggesting that this band may not represent specific binding, but the nonspecific association of a protein with a breakdown product of J8. Overexposure of the autoradiogram depicted in Figure 15 enhanced the presence of this upper band in each lane which contained J8 with HeLa cell extract which alternatively implies that the upper band may be composed of the lower band plus an additional cofactor which is present in nuclear extracts at a low concentration. From these data, either explanation is possible. Binding assays were confirmed with wt and DI leader RNAs, however, J8 consistently bound to a greater degree than wt leader RNA and was used for subsequent binding experiments.

In reactions containing purified proteins, a diffuse band in the lane containing the Ad2 DNA polymerase-pTP complex can be seen near the top of the gel. This may represent significant binding of J8 to one or both of these proteins, but further experiments are needed to determine this point.

J8 Extract Complex Formation with Increasing Amounts of Poly dI/dC.

The specificity of the interaction between J8 and the protein(s) identified on nondenaturing gels was determined. Competition assays were performed according to the procedure of Carthew et al. (1985). Incubations were carried out as before with the addition of increasing amounts of competitor nucleic acid, poly dI(dC). At a 6,000-fold excess (the highest ratio tested) greater than 40% of J8 remained bound indicating a very specific and tight association of J8 with this protein(s) (Figure 16).

Ultraviolet Crosslinking of J8 with a Factor Within HeLa Cell Extracts.

The complex created after incubation of J8 with nuclear extract would dissociates in the presence of SDS making a molecular weight determination difficult. The lack of complex formation upon SDS-polyacrylamide gel electrophoresis (PAGE) is seen in Figure 17. Crosslinking of J8 with the factor, however, might stabilize the complex so that it could be identified upon SDS-PAGE.

Irradiation with ultraviolet light creates covalent linkages between tightly coupled nucleic acid-protein complexes. Coupling reactions occur between purines, pyrimidines and at least 11 different amino acids (Greenberg, 1980). Linkages are specific for the chemical interactions found within the complex.

Figure 16. <u>Competition of Poly dI/dC for Binding in a Nondenaturing Gel</u>. Increasing amounts of poly dI/dC (A) = 0 μg, (B) = 10 μg, (C) = 20 μg, (D) = 40 μg, (E) = 50 μg were added to replication reactions containing 10 ng of ^{32}P radiolabeled J8. After incubation at 31°C for 60 minutes, mixtures were electrophoresed into a 12% nondenaturing polyacrylamide gel and autoradiographed. From densitometric scans of the autoradiogram, in the presence of 60 μg of poly dI/dC, a 6,000-fold excess over the inhibitor, 40% of J8 had remained associated with a protein of the Ad2 infected HeLa nuclear extract. The band observed on this gel is analogous to band II of Figure 15.

J8-EXTRACT COMPLEX FORMATION WITH INCREASING AMOUNTS OF POLY dI/dC

A   B   C   D   E

Figure 17. <u>Ultraviolet Crosslinking Demonstrates the Close Association</u> <u>of J8 to at Least One Protein of 137-Kd of the HeLa Cell Extract.</u> The following substrates were added to replication buffer, incubated for 60 minutes at 31°C, subjected to 12% SDS-PAGE and autoradiographed: (A-D, F) 10 ng of 5' end ^{32}P-radiolabeled J8, (B and C) 60 μg of Ad2 infected HeLa cell nuclear extract. (D) 30 μg of Ad2 infected HeLa cell cytoplasmic extract, (F) 60 μg of uninfected HeLa cell nuclear extract. Lane (E) represents the gel slice of the fast migrating form of J8 (Figure 15, band II) removed from a nondenaturing gel which was U.V.-irradiated for 2 minutes and electrophoresed. After incubation, the reaction mixtures of lanes (C), (D) and (F) were U.V.-irradiated for 2 minutes before electrophoresis.

U.V. CROSSLINKING OF J8 WITH REPLICATION EXTRACT
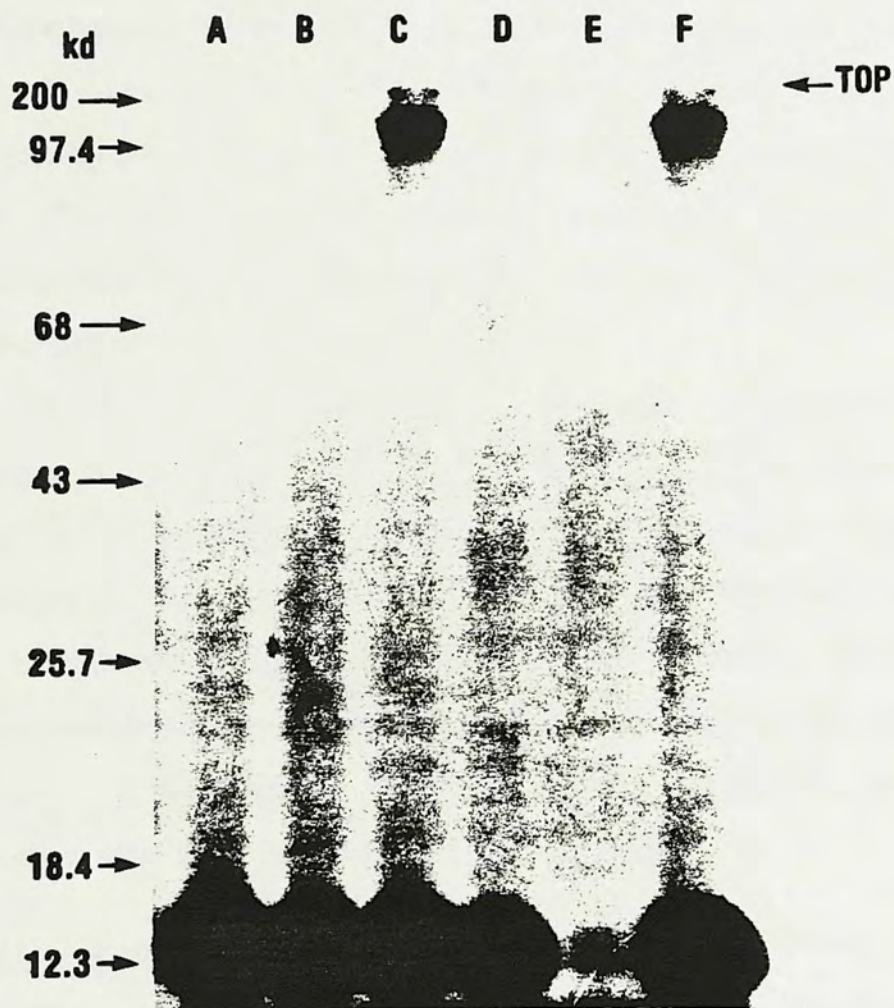
As before, J8 was 5' end-labeled and incubated with 60 μg of Ad2 infected and uninfected HeLa cell extracts. After incubation, mixtures were exposed to shortwave U.V. light (254 nm) for 2 minutes, electrophoresed into a 12% SDS-polyacrylamide gel and autoradiographed (Figure 17). As a result of the crosslinking, a band was observed at approximately 155-Kd which denotes an associated protein(s) of 137-Kd [155-18 (molecular weight of J8)]. This band appears in lanes which contain Ad2 infected and uninfected HeLa cell nuclear extract. Thirty μg of cytoplasmic extract did not bind J8 which may indicate that this protein is not present in the cytoplasm in the proper form or concentration or that there are other factors in the cytoplasm which prevent the association of J8 with this protein.

In an attempt to determine if the complex identified on nondenaturing gels and the 137-Kd protein seen on denaturing gels are the same, a gel fragment containing the J8-protein complex (Figure 15, band II) was cut out of a nondenaturing gel, U.V.-irradiated and subjected to SDS-PAGE. Regretably, no bands were observed. Crosslinking may have been prevented by the presence of acrylamide which will absorb U.V. light. Alternatively, the J8 containing complex may be in such low amounts that it is undetectable in this manner.

Inhibition of pTP-dCMP Complex Formation by J8. As previously discussed, Ad2 DNA replication can be divided into two stages. The first is initiation, covalent attachment of the pTP with dCMP at the origins of replication of the viral genome. The second is elongation, the polymerization of DNA from the 3' hydroxyl residue of dCMP by the Ad2 DNA polymerase. Lichy et al. (1981) have developed an assay to measure the initiation (pTP-CMP complex formation) of Ad2 DNA replication in vitro.

Figure 18.   <u>Inhibition of pTP-dCMP Complex Formation by J8</u>.   pTP-dCMP complex formation reactions were performed as described in Materials and Methods with the following modifications: (A) in absence of Ad2 template, (B) no exogenous nucleic acid, (C) 1.5 ng/μl of J8, and (D) 1.5 ng/μl of J10.   Reaction products were analyzed by 8% SDS-PAGE and autoradiographed.

INHIBITION OF pTP—dCMP
COMPLEX FORMATION

A B C D

80Kd ▶

Briefly, Ad2 template is purified and incubated with the Ad2 infected HeLa cell nuclear extract in the presence of α ^{32}P-dCTP. After incubation, complex (pTP-^{32}P-dCMP) formation was assayed by SDS-PAGE of reaction mixtures and autoradiography. As seen in the autoradiograph depicted in Figure 18, only an 80-Kd protein (pTP) is radioactively labeled. When this experiment is repeated with the addition of J8, pTP-dCMP complex formation was inhibited by 90%. J10 had no affect on the initiation of Ad2 DNA replication in this system. Similar results were obtained with equivalent amounts of wt and DI leader RNAs.

Inhibition and Reconstitution of Ad2 DNA Replication In Vitro Using Purified Protein Fractions. Ad2 DNA replication as described in the Introduction can be performed in vitro using purified proteins, the adenovirus DNA polymerase, the viral DNA binding protein (DBP), the precursor of the pTP, and two host cell proteins, nuclear factors I and II. Replication reactions were performed using purified viral and cellular protein fractions according to the procedure of Ikeda et al. (1981) as described in Materials and Methods. Ad2 DNA template was incubated with the protein fractions containing the Ad2 DNA polymerase-pTP complex, nuclear factor I, and the Ad2 DBP (Table VII). Nuclear factor II is not required for this assay. DNA synthesis observed was found to be specific for Ad2 DNA replication as determined by its dependence on NFI. Titration of J8 in replication reactions resulted in nearly complete inhibition of Ad2 DNA synthesis and confirmed the results obtained in previous experiments using crude extracts. At similar concentrations, J10 had only a small effect.

Experiments identical to these were performed by Mark Kenny at the Memorial Sloan-Kettering Cancer Center which produced consonant 97

Table VII. Ad2 DNA Replication Using Purified Protein Fractions.

Viral Proteins	NFI	J8 (nM)	J10 (nM)	HeLa pol α	Ad2 pol	cpm	pmol	Percent Replication
+	–	––	––	–	–	308	2.1	––
+	+	––	––	–	–	1415	9.4	100
+	+	140	––	–	–	834	5.6	60
+	+	420	––	–	–	604	4.0	43
+	+	700	––	–	–	273	1.8	19
+	+	––	143	–	–	1534	10.2	109
+	+	––	429	–	–	1303	8.7	93
+	+	––	715	–	–	983	6.6	70
+	+	700	––	–	+	2008	13.4	143
+	+	700	––	+	–	965	6.4	68
+	+	––	––	+	–	1174	7.8	83

Ad2 DNA replication reactions were performed as described in Materials and Methods using purified proteins. The viral proteins include the Ad2 DNA polymerase-pTP complex and the Ad2 DBP. The stimulation of Ad2 DNA synthesis observed with the addition of NFI is indicative of Ad2 specific DNA synthesis. The addition of 0.034 units of Ad2 DNA polymerase-pTP (Ad-pol) or 0.8 units of HeLa cell DNA polymerase α-primase complex (HeLa-pol α) restored replication activity to J8 inhibited reactions.

nM, J8 produced 95% inhibition of Ad2 DNA replication while J10 had no effect. In the data presented here, it took 420 nM of J8 to produce 96% inhibition. The inability of J8 to inhibit Ad2 DNA replication at the lower concentration in the experiments reported here may possibly be attributed to the different sources of protein fractions used.

The shut-off of Ad2 DNA replication using crude extracts or purified protein fractions and prevention of pTP-dCMP complex formation by J8 suggested that the target of inhibition may be the Ad2 polymerase-pTP complex. Therefore, additional Ad2 DNA polymerase-pTP complex and HeLa cell DNA polymerase-primase complex were added into J8 inhibited replication reactions. For these experiments, the supplemental proteins were mixed with 250 ng of J8 and incubated at room temperature for 10 minutes before the addition of replication proteins and radioactive label. Replication reactions were then carried out as previously described. The addition of either Ad2 DNA polymerase-pTP or HeLa cell DNA polymerase-primase restored Ad2 DNA replication in this assay (Table VII). To demonstrate that HeLa cell polymerase will not replicate the Ad2 template (Ikeda et al., 1980), purified HeLa cell DNA polymerase-primase complex was added to the Ad2 in vitro DNA replication assay. The addition of 0.8 units of HeLa cell DNA polymerase did not significantly affect Ad2 DNA replication.

The addition of Ad2 DNA polymerase-pTP complex restored replication activity to an inhibited assay, but this result must be interpreted with caution because additional Ad2 DNA polymerase would be expected to increase Ad2 DNA synthesis and does not conclusively prove that J8 is affecting this viral complex. Due to the limited supply of purified proteins, these experiments were not repeated with leader RNA.

The Effects of J8 and J10 on DNA Polymerase Activities In Vitro. The
results obtained with experiments using purified protein fractions
suggest that the target of J8 (or leader RNA) may be the Ad2 DNA
polymerase-pTP, and that other polymerases may also be affected by J8.

Accordingly, a simple assay was used to measure the activities
of the Ad2 DNA polymerase, HeLa cell DNA polymerase and calf thymus
DNA polymerase and the effect of the sequence of VSV leader on the
function of these protein complexes. Activated DNA was prepared by
digestion of calf thymus DNA with pancreatic DNase according to the
procedure of Schlaback et al. (1971). This created nicks and gaps in
double stranded DNA that are recognized by added DNA polymerase to
initiate DNA synthesis. Nucleic acid synthesis was quantitated by acid
precipitation of reaction mixtures containing radiolabeled nucleotides.
DNA polymerase assays were first performed on crude nuclear extracts in
the presence of N-ethylmaleimide (NEM), an inhibitor of eukaryotic DNA
polymerase alpha and Ad2 DNA polymerase (Table VIII). The majority of
DNA polymerase activity was lost in each assay indicating that the
crude extracts contained mostly Ad2 DNA polymerase and DNA polymerase
alpha activities.

Titration of J8 or J10 into assays containing Ad2 infected HeLa
cell nuclear extract and uninfected HeLa cell nuclear extract resulted
in no significant inhibition of activity (Table VIII).

These same experiments were repeated using purified Ad2 DNA
polymerase-pTP, HeLa cell DNA polymerase-primase and calf thymus DNA
polymerase-primase. While J8 had little affect on calf thymus DNA
polymerase, it significantly inhibited both the Ad2 and HeLa cell DNA
polymerases. This result suggests that Ad2 DNA polymerase enzyme is

Table VIII.

Shown is a comparison of the ability of synthetic oligodeoxynucleotides analogous to the sequences of wild-type leader RNA (J8) and DI-011 leader RNA (J10) to inhibit DNA polymerase activities of crude extracts and purified preparations in vitro. The amount of DNA polymerase activity that occurred is given as a percent of DNA synthesis which occurred in the absense of exogenous nucleic acid. For reactions containing NEM, NEM was preincubated with enzyme at the concentration given for 30 minutes on ice prior to the start of the assay.

*Polymerase assays using purified Ad2 DNA polymerase-pTP complex were performed in collaboration with Mark Kenny at the Memorial Sloan-Kettering Cancer Center, New York.

Table VIII. The Effect of J8 and J10 on DNA Polymerase Activities.

DNA Polymerase Tested	N-ethyl-maleimide	J8 (nM)	J10 (nM)	Reaction Products cpm	Reaction Products pmol	Percent of control
Ad2 infected HeLa cell nuclear extract	--	--	--	15785	110.8	100
	10 mM	--	--	564	3.6	2
		45	--	18543	116.8	105
		90	--	17609	110.9	100
		225	--	18657	117.5	106
		--	46	16234	102.3	92
		--	92	17370	109.4	99
		--	230	16684	105.1	95
Uninfected HeLa cell nuclear extract	--	--	--	22445	147.7	100
	10 mM	--	--	375	2.4	2
		45	--	21185	133.5	90
		90	--	23851	150.3	102
		225	--	22661	142.8	97
		--	46	21876	137.8	93
		--	92	21786	137.3	93
		--	230	21521	135.6	92
Ad2 DNA polymerase-pTP complex*		--	--	13730	1.84	100
		56	--	7504	0.94	53
		168	--	4832	0.58	33
		560	--	3300	0.37	21
		--	57	12912	1.73	94
		--	171	12230	1.64	89
		--	570	10134	1.36	73
HeLa cell DNA polymerase-primase complex		--	--	1703	10.8	100
		45	--	1075	6.8	63
		90	--	762	4.8	45
		225	--	565	3.6	33
		--	46	1637	10.3	96
		--	92	1599	10.1	93
		--	230	1689	10.7	99
Calf thymus DNA polymerase-primase complex		--	--	5222	32.9	100
		45	--	5214	32.9	100
		90	--	5235	33.0	100
		225	--	3920	24.7	75
		--	46	4851	30.6	93
		--	92	4935	31.1	95
		--	230	4502	28.4	86

most affected during Ad2 DNA replication in vitro. The inability of J8 to prevent calf thymus DNA polymerase activity indicates that the inhibition of Ad2 and HeLa cell DNA polymerase activities is not caused by a nonspecific toxic effect. The results obtained were not confirmed with leader RNA.

Discussion

The ability of VSV to shut-off host cellular macromolecular synthesis was initially recognized by Wagner and Huang in 1965 (1966). They were the first investigators to report that the exceptional virulence of VSV was associated with the inhibition of cellular DNA, RNA and protein synthesis in infected Krebs-2 carcinoma cells. These same authors in a later publication reported that U.V.-irradiation of VSV does not affect its ability to inhibit cellular RNA synthesis and erroneously suggested that the proteins of the infecting virions were themselves cytotoxic and responsible for shut-off (Huang and Wagner, 1965). Yaoi et al. (1970) repeated these studies and confirmed the observations of Huang and Wagner. They did not speculate on the cytotoxicity of infecting virions, but simply stated that a U.V.-resistant component of the virus was responsible for shut-off.

These three reports form the foundation on which a great deal of research lies that deals with the phenomenon of VSV induced host cell shut-off. Until 1981, they were also the only published studies which dealt with the VSV induced inhibition of host DNA synthesis. There have been a large number of studies published on the effects of VSV on RNA synthesis. This disproportion may have arisen because researchers believed that VSV directly inhibited only RNA and protein synthesis and that the inhibition of DNA synthesis was the indirect result (Yaoi et al., 1970; Kranz and Reichmann, 1983; Wagner and Huang, 1966).

Studies have shown that active eukaryotic replication requires continual protein and RNA synthesis (Ginsberg, 1980). Cells do not maintain large pools of histones but require constant translation of

histone mRNA. If histone translation is halted, DNA replication abruptly stops. RNA synthesis is required in the process of primer formation by DNA primase and possibly for histone mRNA synthesis as well. Kranz and Reichman (1983) investigated the inhibition of SV40 replication by VSV in doubly infected monkey kidney cells. They concluded that VSV inhibited active protein synthesis which is required for SV40 replication but could not rule out any direct effects of VSV on SV40 DNA synthesis. Presently, there is no way to separate the processes of DNA and RNA synthesis in eukaryotic cells, and there are no VSV mutants in which only DNA or RNA synthesis is inhibited making the study of the effect of VSV on DNA synthesis arduous.

McGowan and Wagner (1981) analyzed the phenomenon of VSV induced shut-off of DNA synthesis in MPC-11 and L-cells. They first determined that U.V.-irradiation of VSV (20,000 ergs/mm^2) sufficient to impair transcription of all viral products except leader RNA did not significantly affect the inhibition of DNA synthesis in these cells. At U.V. doses which would affect transcription of leader RNA, the ability of VSV to inhibit DNA synthesis was reduced.

These same authors established the kinetics of inhibition of DNA and RNA synthesis of cell cycle synchronized MPC-11 cells. The rates of extent of inhibition of DNA and RNA synthesis were found to be almost identical. This result would not be expected unless VSV was able to directly inhibit both processes. They went on to determine that VSV did not restrict uptake of deoxynucleoside triphosphates or degrade existing DNA. Alkaline sucrose gradient analysis of pulse labeled DNA from VSV infected cells demonstrated that elongation of already initiated chains of DNA was not affected. Comparison of non-

specific DNA polymerase and thymidine kinase activities of cell free extracts prepared from VSV infected cells and mock infected cells showed that VSV had no effect. Their results did not rule out all possible effects of VSV on DNA polymerase activity or on the initiation of DNA synthesis nor could they quell the possibility that VSV only inhibited DNA synthesis indirectly through the direct inhibition of host RNA synthesis.

The specific aim of this dissertation was to determine if VSV can directly inhibit DNA synthesis. These studies were possible because of the development of the Ad2 in vitro replication assay by Challberg and Kelly (1979a). This assay is advantageous for a number of reasons. First, adenovirus replication in vitro requires cellular proteins and machinery and is believed to mimic adenovirus DNA synthesis in vivo. Second, adenovirus replication initiates by the covalent binding of a viral protein to dCMP at the terminus of the viral genome and does not require RNA primer formation. Therefore, for the first time, the ability of VSV to inhibit DNA synthesis can be determined in the absence of any effects of VSV on RNA synthesis.

The results presented have shown that coinfection of VSV into Ad2 infected HeLa cells will shut down both adenovirus and cellular protein synthesis. Purified VSV leader RNA and synthetic deoxynucleotides analogous to the sequence of leader will inhibit the specific replication of Ad2 DNA in vitro both in the crude reaction and in a system using purified components. Oligo(dT) selected VSV mRNA, nonspecific RNA and other synthetic deoxynucleotides not related to the sequence of wt leader did not inhibit Ad2 replication. Leader RNA was found to prevent the initiation event required for replication, the

covalent joining of the precursor of the Ad2 pTP to dCMP at the ends of the adenovirus genome. Initiation requires the enzymatic functions of the Ad2 DNA polymerase, the precursor of the Ad2 pTP, the Ad2 DBP and HeLa cell NFI. Leader RNA made from defective interfering particle 011, which does not inhibit host cellular macromolecular synthesis in vivo and is 61% homologous in sequence with the leader sequence has no inhibitory effects on Ad2 in vitro replication or pTP-dCMP complex formation. Reconstitution of replication was observed when supplemental Ad2 infected or uninfected HeLa cell nuclear extract was added to inhibited reactions. Cytoplasmic extract from Ad2 infected HeLa cells which is rich in viral proteins could not reverse an inhibited reaction.

The ability of VSV leader RNA to inhibit nucleic acid synthesis was first postulated by Weck et al. (1979). U.V.-irradiation of VSV, sufficient to prevent transcription of all viral messages except leader RNA prior to infection, resulted in shut-off of host cell RNA synthesis comparable to shut-off produced by unirradiated VSV. McGowan et al. (1982) provided direct evidence that leader RNA inhibits eukaryotic RNA synthesis. Using a eukaryotic in vitro transcription assay, they determined that purified leader RNA will inhibit transcription of RNA polymerase II and RNA polymerase III genetic elements in vitro. Despite the criticism that the in vitro system may not accurately reflect the events which occur in vivo, the authors had established a direct link between VSV leader RNA and the process of eukaryotic transcription.

McGowan et al. (1982) speculated that leader may act as a surrogate for other cellular derived small RNAs that regulate metabolism. Kurilla and Keene (1983) found that VSV leader RNA associated with La protein, a cellular protein which is typically found associated with

precursors of RNA polymerase III transcripts. La antiserum precipitated leader RNA with La protein as a ribonucleoprotein complex from VSV infected BHK cells indicating that leader RNA associates with at least one host protein in vivo.

La antiserum is derived from human patients with systemic lupus erythematosis, a fatal autoimmune disorder in which the patients' immune system mounts an antibody response against their own cellular components, one of which is the La protein. La protein has been shown to selectively bind the 3' uridylate residues of mRNA and was proposed to be a transcription termination factor (Gottlieb and Steitz, 1986). One may reason that the interaction between VSV leader RNA and the La protein creates a transcription terminator which is responsible for the shut-off of RNA synthesis in the infected cell. Kurilla et al. (1984) found that La antisera will coprecipitate La protein and leader RNA from rabies virus infected BHK-21 cells. Rabies virus leader RNA is 58 n.t. in length and 59% homologous to regions of the sequence of VSV leader RNA. Rabies virus, in contrast to VSV, has no effect on macromolecular synthesis of the infected cell. From these studies, it is clear that the function of La protein within the cell must first be determined before any conclusions may be drawn from its interaction with leader RNA in the inhibition of cellular macromolecular synthesis.

VSV leader RNA rapidly accumulates in the nucleus of infected BHK cells (Kurilla et al., 1982). This accumulation corresponds with the initiation of maximal inhibition of host cell macromolecular synthesis. Grinnell and Wagner (1983) established the number of leader RNA molecules per VSV_{IND} infected MPC-11 cell by hybridization of total cellular RNA to 3' end radiolabeled VS virion RNA. At 4-5 hours p.i.,

it was determined that at least 2500 copies of leader RNA are present in each infected cell.

Accumulation of leader RNA was not observed in L-cell populations persistently infected with VSV (Wilusz et al., 1985). Wilusz et al. (1985) sequenced the 3' ends of VSV genomes isolated from three independently derived, persistently infected L-cell populations and determined that the 3' untranslated region which codes for leader RNA contained single base mutations suggesting an involvement of leader RNA in the establishment of persistence. Further studies of the viral strains isolated from persistently infected L-cell cultures (Frey and Youngner, 1984) identified strains that inhibit host cellular RNA and protein synthesis more slowly than does wt VSV. These mutant strains also synthesize 6 to 8-fold less leader RNA than wt VSV.

McGowan et al. (1982) determined that purified VSV leader RNA would inhibit RNA polymerase II and III transcription in vitro. They reasoned that two regions present in the wt, but not in the DI leader RNA were important for the inhibitory activity observed. These two regions include an AU rich "TATA"-like sequence (n.t. 18-29) and a purine rich sequence (n.t. 31-44). These sequences have been found to be highly conserved in all strains of VSV isolated.

A comparison of the conserved regions of the adenovirus origin of replication with VSV leader RNA reveals some analogies (Figure 19). The 3' termini of both the VSV and Ad2 genomes are thought to be the entry sites of the viral polymerases and contain the sequence information necessary for accurate and efficient replication. The sequence data in Figure 19 shows that in both VSV leader RNA and the 5' terminal sequences of the Ad2 genome are found a tri-nucleotide repeat of CAA/T

followed by a AT (AU) rich region then an AG rich region. Examination of the origins of replication of polyoma, BK and SV40 viruses reveals similar homologies not found in the sequence of DI leader RNA. These homologies may be coincidental, but the sequences themselves are individually important for replication and highly conserved in each strain of virus.

Grinnell and Wagner (1985) analyzed the inhibition of eukaryotic transcription in vitro using synthetic oligodeoxynucleotides analogous to different regions of leader. They found that the central AU sequence of leader RNA (n.t. 18-24), or its deoxynucleotide homologue were the minimal requirements for inhibition of in vitro transcription by RNA polymerase II or III. Additional sequences flanking this region increase its inhibitory activity. This AU rich region of leader is homologous (n.t. 19-25) with a sequence in the AT rich area of the Ad2 origin of replication (n.t. 19-25). This region of the Ad2 genome is within the NFI binding site and adjacent to the proposed Ad2 DNA polymerase binding site.

A series of synthetic oligodeoxynucleotides analogous to small regions of VSV leader RNA were tested for their ability to inhibit Ad2 DNA replication in vitro. Synthetic molecules which contained the 3' terminal AG-rich nucleotides (n.t. 41 to 36), J2, or the central AU-rich nucleotides (n.t. 17 to 33), J1, were not able to significantly prevent Ad2 specific DNA synthesis. Only the synthetic oligodeoxynucleotide that spanned n.t. positions 8 to 29 (J5) was able to affect Ad2 DNA replication. J2, at all concentrations tested, inhibited Ad2 DNA replication by 20% which suggests that inhibition is occurring by a nonspecific mechanism unrelated to the mechanism of inhibition of VSV

113

Figure 19. A Comparison of the Sequence of Wt Leader RNA with Eukaryo-
tic and Viral Sequences that May be Functionally Related. Shown are
(Ad2) the 5' terminal sequence of adenovirus serotype 2; (Wt-L) the
sequence of wt VSV leader RNA (Colonno and Banerjee, 1978); (DI-L) the
sequence of the defective interfering particle DI-011 small RNA, DI
leader RNA (Shubert et al., 1978); (Pol II) the consensus sequence
required for accurate and efficient transcription of cellular mRNA by
eukaryotic RNA polymerase II (Baker and Ziff, 1981); (Pol III) a se-
quence identified by Galli et al. (1981) to be conserved in possible
promoter regions for genes transcribed by eukaryotic RNA polymerase
III; sequences found at the origins (Jelinek et al., 1980) and im-
plicated as necessary for the replication of (SV40) simian virus strain
40; (BKV) human BK virus and (HBV) human hepatitis virus; (Enhancer) a
sequence reported by Laimins et al. (1983) to be present in possible
eukaryotic enhancer elements (NFI). The proposed recognition sequence
required for NFI binding (Philip Rosenfeld, personal communication).
All sequences are presented in their 5' to 3' orientation. The region
of the Ad2 genome required for nuclear factor I binding is indicated
with a broken line and the region required for Ad2 DNA polymerase with
a continuous line.

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Ad2      5'-CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAG-3'

Wt-L     5'-ACGAAGACAAACAAACCAUUAUUAUCAUUAAAAGGCUCAGGAGAAAA   -3'

DI-L     5'-ACGAAGACCACAAAACCAGAUAAAAAAUAAAAACCACAAGAGGGUC   -3'

Pol II   ("TATA" BOX)            TATATAAT

Pol III                                 GGTGGCNNAGTGGT

SV40                                    AGAGGCCGAGGCGG

BKV                                     GGAGGCAGAGGCGG

HBV                                     GGAGGCAGAGGCGG

Enhancer (consensus sequence)            TGGAAAG

NFI (consensus sequence)            TGGANNNNNGCCA
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leader RNA. J5, which produced the largest amount of inhibition, spans the region of VSV leader RNA which is analogous to the entire sequence of the Ad2 DNA polymerase binding site on the Ad2 template. This region of VSV leader RNA has no significant homology to the sequence of rabies virus leader RNA and rabies virus does not inhibit host cell macromolecular synthesis (Kurilla et al., 1984).

J5 contains the 7 nucleotides which are homologous between VSV leader RNA (n.t. 19 to 25) and the Ad2 DNA 5' terminal sequence (n.t. 19 to 25) and most of the sequence identified by Grinnell and Wagner (1985) as being important for inhibition of RNA transcription in vitro (n.t. 16 to 34). Again, this region of VSV leader is linked to an inhibitory function of the RNA, but why? Obviously, it could not code for a peptide. It may hybridize to sequences of DNA or RNA in a manner similar to the U1 RNA which has been shown to be involved in RNA splicing (Setyono and Pederson, 1984). This small nuclear RNA exists within a ribonucleoprotein complex, and it is this complex which recognizes the mRNA molecule. Both U1-mRNA and U1-protein interactions are required for a stable and functional association to occur. It is logical to assume that VSV leader RNA is also binding to a protein or nucleic acid, and it is this interation which results in the inhibition of RNA transcription and A2 DNA replication in vitro.

Results presented here using sucrose gradients and nondenaturing, low ionic strength acrylamide gel electrophoresis demonstrated a specific association of the synthetic oligonucleotide J8 (analogous to the sequence of VSV leader RNA) with proteins of HeLa cell nuclear and cytoplasmic extracts. These proteins were resolved as a faster migrating complex and a slower migrating complex. These complexes

exist in Ad2 infected and uninfected HeLa cell nuclear extracts as well as Ad2 infected HeLa cell cytoplasmic extract but to a lesser degree. J8-protein complexes observed in nondenaturing gels consistently comigrated with each extract. Under limiting amounts of J8, the faster migrating complex bound all available label. Only when J8 was in excess would the slower migrating complex be able to form. It may be that the faster and slower migrating complexes observed are the same proteins. Their size differences may reflect the binding of an associated factor. Binding competition assays showed that in the presence of a 6,000-fold excess of poly dI/dC, J8 remained bound to the faster migrating protein on nondenaturing gels. This could only occur if there was a tight and specific bond between the two molecules. J10 also bound a protein in nuclear extracts, and the complex formed comigrated with the J8-protein complex on nondenaturing gels. These experiments, however, do not differentiate functional from nonfunctional binding.

U.V.-irradiation of replication mixtures containing J8, crosslinked this synthetic to a protein which was sized by SDS-PAGE to approximately 137-Kd. U.V.-irradiation will crosslink nucleotides with amino acids and amino acids with each other, therefore, it could not be determined from these experiments if this protein - DNA complex contains a single polypeptide of 137-Kd, a multimer of 2 or more identical proteins or a small number of different proteins. It is likely though that this protein(s) is one or more of the proteins involved with Ad2 replication, and that its association with J8 (or leader RNA) results in the observed inhibition of Ad2 DNA replication. These proteins are the Ad2 DNA polymerase, the Ad2 pTP, the Ad2 DBP and HeLa cell NFI.

NFI is a 47-Kd cellular protein that was first identified for its ability to stimulate the initiation of Ad2 replication in vitro (Nagata et al., 1982). DNase analysis has shown that NFI protects a 19 n.t. region of the origin of Ad2 replication which includes the highly conserved AT-rich region (see Figure 19). These studies suggested that NFI acts as a dimer on the Ad2 template. If so, it is possible that the complex created on SDS-PAGE of U.V.-irradiated replication reaction containing J8 may include a multimeric form of NFI.

Recently, NFI has been found to bind to the "CAT" box, a sequence of nucleotides typically in the order CCAAT which is important for accurate and efficient transcription of RNA polymerase II genetic elements (George Khoury, personal communication). NFI binding sites have been identified in the human genome, the mouse mammary tumor virus - long terminal repeat, and the BK virus enhancer, a transcriptional activator (Nowock et al., 1985).

If NFI is both a transcription and a replication factor, it would be possible for VSV to inhibit both processes by inactivating this one protein. Also, NFI is the only host protein required for Ad2 DNA replication in vitro. VSV leader RNA does not contain the consensus sequence required for NFI binding (see Figure 19), and NFI did not reconstitute inhibited Ad2 DNA replication in vitro. So why did the addition of Ad2-infected and uninfected HeLa cell nuclear extract restore Ad2 specific replication in vitro? Reconstitution observed using these crude extracts may have been due to the introduction of additional RNases, inherent to the extracts which digested leader. This seems unlikely because cytoplasmic extract which would also con-

tain these RNases did not restore Ad2 replication. As was observed on nondenaturing gels, a protein present in Ad2 infected and uninfected HeLa cell nuclear extracts has a very high affinity for the sequence of leader RNA. This protein is seen in cytoplasmic extracts but in much lower amounts. More likely, is that the extracts were able to provide additional proteins or factors which bound leader RNA and allowed Ad2 replication to proceed. The ability of the sequence of leader to bind to a protein found in Ad2 infected and uninfected nuclear extracts suggests that it is a cellular protein. There is no unequivocal way to demonstrate this possibility until the proteins involved are identified and characterized.

Among the Ad2-derived possible targets of leader RNA is the Ad2 DBP. The DBP's functions during replication are somewhat of a mystery. Reconstruction of replication in vitro using purified protein fractions absolutely requires the presence of the DBP (Ikeda et al., 1981). In contrast, nuclear extracts prepared at the nonpermissive temperature from HeLa cells infected with a ts DBP⁻ mutant of Ad2 will carry out replication in vitro (Kaplan et al., 1979). A cellular DNA binding protein may be substituting for the Ad2 DBP in vivo and in vitro. It has been determined that the DBP is not required for initiation, but functions during elongation of already initiated chains (Challberg et al., 1982). The sequence of leader inhibits the initiation of Ad2 replication making it unlikely that the DBP is the target.

The other viral proteins required for Ad2 replication are the adenovirus DNA polymerase and the pTP. The Ad2 DNA polymerase enzyme is biochemically similar to eukaryotic DNA polymerase alpha. Both have the same temperature optimums, cation requirements, salt requirements

and sensitivity to NEM. The principal difference between the two polymerases is that Ad2 DNA polymerase is ten times more resistant to aphidicolin than is DNA polymerase alpha (Lichy et al., 1982).

Aphidicolin, however, will inhibit Ad2 DNA replication both in vivo and in vitro (Longiaru et al., 1979). This characteristic was taken as evidence that eukaryotic DNA polymerases alpha or beta was involved with Ad2 DNA replication. Recent experiments, though, have found that aphidicolin sensitivity is only observed when the Ad2 DNA polymerase functions together with the Ad2 DBP, pTP, NFI and NFII (Nagata et al., 1983). pTP-dCMP complex formation was unaffected by aphidicolin suggesting that its effect is during elongation of full-length template DNA. Further studies are required in order to fully understand the mechanism of inhibition of aphidicolin.

The results presented here imply that the Ad2 DNA polymerase-pTP complex may be the target of leader RNA (or J8) in the inhibition of Ad2 replication. J8 inhibits the initiation event of Ad2 replication which requires the Ad2 DNA polymerase-pTP complex and the addition of supplemented Ad2 DNA polymerase-pTP complex into an inhibited replication reaction restored specific Ad2 DNA synthesis. Furthermore, J5, the synthetic oligodeoxynucleotide which contains the sequences of leader that are analogous to the sequence of the Ad2 DNA polymerase binding site, produced the largest degree of inhibition of Ad2 replication. Both B17 and B19 which contain part of this sequence produced substantial inhibition. Although J8 could not prevent nonspecific Ad2 DNA polymerase activity in crude extracts, it would inhibit polymerase activity of purified Ad2 DNA polymerase-pTP complex in in vitro assays (Mark Kenny, personal communication).

These data do not indicate whether the Ad2 DNA polymerase, the Ad2 pTP or the complex of these two proteins is directly affected by leader RNA. Since J8 prevents dCMP-pTP complex formation (initiation), it is possible that the function of the pTP is affected. The Ad2 pTP (as is eukaryotic DNA primase) is tightly coupled to its associated DNA polymerase. The two polypeptides can be separated on urea gels, but Ad2 replication using purified isolates of the Ad2 DNA polymerase and the pTP has not been reported.

The complex created between J8 and a protein(s) of HeLa cell extracts may contain the Ad2 DNA polymerase. The apparent size of this viral protein polymerase was determined by sedimentation analysis to be 140-Kd, very close to the molecular weight of the protein identified on gels which was U.V. crosslinked to J8 in HeLa cell extracts. J5, the small synthetic oligodeoxynucleotide, which produced the largest degree of inhibition of specific Ad2 DNA synthesis, contains the nucleotides analogous to VSV leader RNA which are similar to the Ad2 terminal sequence that is the binding site for the Ad2 DNA polymerase. This result also suggests that the leader sequence may be interfering with Ad2 DNA polymerase binding to the Ad2 template, possibly by binding to the polymerase itself.

The complexes formed upon incubation of J8 with Ad2 infected HeLa cell nuclear extract and uninfected nuclear extract on nondenaturing gels appear to comigrate suggesting that they contain the same proteins. Also, uninfected nuclear extract restored Ad2 replication in vitro which had been inhibited by leader RNA. It was consistently observed that in the same amounts of Ad2 infected or uninfected extract, there was a greater amount of J8 protein complex formation on nonde-

naturing gels and a greater degree of reconstitution using Ad2 infected nuclear extract. It may be that the Ad2 polymerase does bind to the sequence of leader RNA, but comigrates with a cellular protein which also binds J8. Two-dimensional gel electrophoresis may resolve this question. Another eukaryotic protein, functionally related to the Ad2 DNA polymerase may be the quaesitum of VSV leader RNA and responsible for the inhibition of host nucleic acid synthesis in VSV infected cells.

This ultimate target of J8 or leader RNA may be eukaryotic DNA polymerase alpha. Although DNA polymerases beta and gamma are also present in nuclear extracts, DNA polymerase alpha is responsible for 98% of the DNA synthesis activity observed in extract preparations (see Table VIII). This would explain why reconstitution was observed of inhibited Ad2 replication reactions with the addition of supplemented Ad2 infected and uninfected HeLa cell nuclear extract. Excess DNA polymerase alpha bound leader RNA preventing its inhibition of the Ad2 DNA polymerase.

As has been shown in the Results, leader RNA inhibits the specific replication of Ad2 terminal sequences in the in vitro assay using crude extract, but it also inhibits nonspecific DNA synthesis which is carried out by HeLa cell DNA polymerase alpha, (Philip Rosenfeld, personal communication). Experiments performed during the course of this project, but not directly related, showed that leader RNA would inhibit DNA synthesis of any DNA template incubated in the replication extract (data not shown). This result may be attributed to the inhibition of eukaryotic DNA polymerase alpha. In vitro DNA polymerase assays using purified HeLa DNA polymerase alpha-DNA primase complex

showed that J8 drastically inhibited DNA synthesis while J10 had little affect at the same concentration, and reconstitution experiments with purified HeLa cell DNA polymerase-primase complex were successful. This could only be explained if the added protein bound or associated with the sequence of leader, thereby preventing its interaction and consequential inhibition of the Ad2 DNA polymerase-pTP complex because HeLa cell DNA polymerase will not replicate Ad2 DNA (see Table VII).

HeLa cell DNA polymerase alpha, purified on potassium phosphate gradients which does not eliminate all accessory proteins is from 110 to 180-Kd in size (Holmes et al., 1983) suggesting that the major J8 binding protein identified in nuclear extracts may be DNA polymerase alpha. The slow migrating complex observed in cytoplasmic extracts could be a composite of J8, the DNA polymerase and the DNA primase. Eukaryotic DNA polymerases and DNA primases are typically copurified as tightly bound complexes which can only be separated by proteolysis of one polypeptide or the other. DNA primase was recently isolated from HeLa cells by Gronostajski et al. (1984), but no molecular weight was given. DNA primase activities isolated from other cell types are typically in the range of 48 to 110-Kd (Pelevani et al., 1984; Holmes et al., 1986).

If the Ad2 pTP is affected by leader RNA in vitro, the eukaryotic DNA primase enzyme may be the target of leader during VSV infection in vivo. If so, this could explain how leader RNA is able to shut-off both DNA and RNA synthesis by inactivating DNA primase and RNA polymerase. Leader RNA may bind to their active sites preventing RNA synthesis. Using this same argument, leader RNA may be directly effecting the DNA polymerase enzyme. Both DNA polymerase and RNA poly-

merase perform analogous functions, synthesis of nucleic acid. Again, leader RNA could be binding to the active sites of these molecules preventing DNA and RNA synthesis. This would explain why both VSV leader RNA and synthetic DNA analogous to the sequence of leader are able to inhibit nucleic acid synthesis. The structures of leader RNA and leader DNA may be indistinguishable to these enzymes.

To fully predicate the inhibitory functions of VSV leader RNA, sophisticated enzymatic experiments must be performed. Only recently have the major polypeptides of a eukaryotic DNA polymerase-primase been identified, and their functions sorted between the DNA polymerase and the DNA primase (Holmes et al., 1986). Final resolution of the process by which leader RNA is able to effect these polypeptides should prove interesting to both virologists and enzymologists.

Conclusion

In summary, it is clear that VSV leader RNA will directly inhibit DNA synthesis in both Ad2 in vitro replication reactions and nonspecific DNA polymerase assays using either Ad2 DNA polymerase or HeLa cell DNA polymerase. Inhibition may be the result of a sequence specific interaction of leader RNA with the DNA polymerase enzyme or a closely associated factor which regulates polymerase activity, namely, eukaryotic DNA primase or the Ad2 pTP.

The study of the effects of VSV leader RNA on Ad2 in vitro replication has proceeded as far as current adenovirus technology will allow. Further investigations of leader should explore the mechanism of inhibition of DNA polymerase activity in order to more fully understand the functions of this small viral RNA and the complex process of DNA synthesis.

Some of the more interesting questions concerning the inhibitory process of VSV are as yet unanswered. How is leader RNA able to inhibit both RNA transcription and DNA replication? Does the inhibitory activity of leader RNA in vitro mimic the function of leader RNA in vivo? How is leader RNA involved with the inhibition of host cell translation in VSV infected cells? What other advantages does leader RNA confer to the virus which would account for its highly conserved sequence?

Collaborations were initiated with a wide spectrum of scientists around the country to try to answer some of these questions. Researchers, studying the processes of SV40 and eukaryotic mitochondrial replication, are currently testing the effects of leader RNA on their in vitro systems. Their results will provide a functional means to explore the differences and similarities between the Ad2 DNA poly-

merase and the SV40 and mitochondrial DNA polymerases.

Finally, the effects of VSV leader RNA must be established in vivo. Again, collaborations were begun, but unfortunately not completed at the conclusion of this dissertation. In one experiment, large numbers of leader RNA molecules are to be packaged into lipid vesicles, fused with cells in tissue culture and their effect on macromolecular synthesis analyzed. A second collaborator has cloned the sequences of the wt and DI-011 leader RNA into bovine papilloma virus shuttle vectors downstream of a controllable promoter. Upon introduction of these vectors into appropriate cells and stimulation of transcription of the sequence of leader RNA, its effect on cellular nucleic acid and protein synthesis can be determined.

In conclusion, the study of the effect of VSV leader RNA on Ad2 replication has opened the door for future studies of the function of this small viral RNA which should lead to thoughtful insights into the processes of virus-host cell interactions and the most basic aspects of replication, transcription and translation.

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